

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published: _____

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)



**(19) World Intellectual Property Organization
International Bureau**



(43) International Publication Date
5 April 2001 (05.04.2001)

72)	Investigator	KREIG, Arthur M.	University of Iowa, Dept. of Internal Medicine, 404 EM Bldg, Iowa City, IA 52242	US, SCHEFFER, Carolee; Quagen GmbH, Quagen Pharmaceuticals, 41-D-40724 Hilden, Germany; VOLLMEIER, Jörg; Colony Pharmaceutical Group, 41-D-40724 Hilden, Germany	Quagen GmbH, Quagen GmbH, Max-Planck-Institut für Immunologie, 41-D-40724 Hilden, Germany
73)	International Patent Classification(s)	A61K 31/7088, 39/59, 4/803, 3/712, A2L 1/00, A61P 3/704	US, A61P 3/704	A61K 31/7088, 39/59, 4/803, 3/712, A2L 1/00, A61P 3/704	A61K 31/7088, 39/59, 4/803, 3/712, A2L 1/00, A61P 3/704
74)	International Application Number	PC705002933			

21) International Application Number PCT/US00/26383

(22) International Filing Date: 23 September 2000 (25.09.2000)
(74) Agent: LOCKHART, HENR, C.; WOLF, Greenfield & Sachs, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

259 Filling Leinhardt

260 Publication Law and Ethics

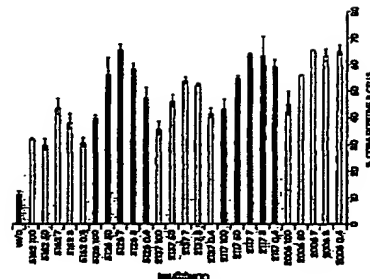
30) Priority Data

60/156,135	27 September 1999 (27.09.1999)	09
60/227,436	21 August 2000 (21.08.2000)	1/9

[illegible]

(Continued on next page)

350 THE IMMUNOSTIMULATORY NUCLEIC ACIDS



(37) Albrectt The invention relates to immunomodulatory nucleic acid compositions and methods of using the compositions. The T-rich nucleic acid compositions contain poly T sequences and/or have greater than 24% T nucleotide residues. The T₁₀ nucleic acids have T₁₀ dinucleotides. The C-rich nucleic acids have at least one poly-C region which is greater than 30% C nucleotides. These immunomodulatory nucleic acids function in a similar manner to nucleic acids containing CpG motifs. The invention also encompasses preferred CpG nucleic acids.

WO 01/22972 A2

IMMUNOSTIMULATORY NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

Bacterial DNA has immune stimulatory effects to activate B cells and natural killer cells, but vertebrate DNA does not (Tokumaga, T., et al., 1988, *Jpn. J. Cancer Res.* 79:682-686; Tokumaga, T., et al., 1984, *JNCI* 72:955-962; Manning, J.P., et al., 1991, *J. Immunol.* 147:1759-1764; and reviewed in Kriegl, 1998, *Int. Applied Oligonucleotide Technology*, C.A. Stein and A.M. Kriegl, (Eds.), John Wiley and Sons, Inc., New York, NY, pp. 431-449). It is now understood that these immune stimulatory effects of bacterial DNA are a result of the presence of unmethylated CpG dinucleotides in particular base contexts (CpG motifs), which are common in bacterial DNA, but methylated and underrepresented in vertebrate DNA (Kriegl et al., 1993 *Nature* 374:346-349; Kriegl, 1999 *Biochim. Biophys. Acta* 932:1-10). The immune stimulatory effects of bacterial DNA can be mimicked with synthetic oligodeoxynucleotides (ODN) containing these CpG motifs. Such CpG ODN have highly stimulatory effects on human and murine leukocytes, inducing B cell proliferation, cytokine and immunoglobulin secretion, natural killer (NK) cell lytic activity and IFN- γ secretion, and activation of dendritic cells (DCs) and other antigen presenting cells to express costimulatory molecules and secrete cytokines, especially the Th1-like cytokines that are important in promoting the development of Th1-like T cell responses. These immune stimulatory effects of native phosphodiester backbone CpG ODN are highly CpG specific in that the effects are essentially abolished if the CpG motif is methylated, changed to a CpC, or otherwise eliminated or altered (Kriegl et al., 1993 *Nature* 374:346-349; Hartmann et al., 1999 *Proc. Natl. Acad. Sci. USA* 96:9305-10). Phosphodiester CpG ODN can be formulated in lipids, alum, or other types of vehicles with depot properties or improved cell uptake in order to enhance the immune stimulatory effects (Yamamoto et al., 1994 *Microbiol. Immunol.* 38:831-836; Granuzinski et al., 1998 *Mol. Med.* 4:109-118).

In early studies, it was thought that the immune stimulatory CpG motif followed the formula purine-purine-CpG-pyrimidine-pyrimidine (Kriegl et al., 1993 *Nature* 374:346-349; Flieschly, 1996 *J. Immunol.* 156:421-423; Backer et al., 1998 *EMBO J.* 17:6230-6240; Lifjard et al., 1998 *Trends in Microbiol.* 6:496-500). However, it is now clear that mouse lymphocytes respond quite well to phosphodiester CpG motifs that do

-2-

not follow this "formula" (Yi et al., 1998 *J. Immunol.* 160:3898-3906) and the same is true of human B cells and dendritic cells (Hartmann et al., 1999 *Proc. Natl. Acad. Sci. USA* 96:9305-10; Liang, 1996 *J. Clin. Invest.* 98:1119-1129).

Several past investigators have looked at whether the nucleotide content of ODN may have effects independently of the sequence of the ODN. Interestingly, antisense ODN have been found to be generally enriched in the content of GG, CCC, CC, CAC, and CG sequences, while having reduced frequency of TT or TCC nucleotide sequences compared to what would be expected if base usage were random (Sniderman et al., 1996 *Antisense Nucleic Acid Drug Develop.* 6:63-67). This raised the possibility that the over-represented sequences may comprise preferred targeting elements for antisense oligonucleotides or *vis a versa*. One reason to avoid the use of thymidine-rich ODN for antisense experiments is that degradation of the ODN by nucleases present in cells releases free thymidine which competes with 3H-thymidine which is frequently used in experiments to assess cell proliferation (Mason et al., 1992 *Antisense Research and Development* 2:325-330).

SUMMARY OF THE INVENTION

The present invention relates in part to pyrimidine rich (Py-rich) and in some embodiments thymidine (T) rich immunostimulatory nucleic acids which do not require the presence of a CpG motif. The present invention also relates in part to the discovery that nucleic acids which contain a TG dinucleotide motif are also immunostimulatory. The invention is based in part on the unexpected finding that nucleic acid sequences which do not contain CpG motifs are immunostimulatory. It was discovered upon analysis of the immune stimulation properties of many nucleic acid sequences that these sequences may be Py-rich e.g., T-rich or that they may contain TG motifs. It was also discovered that these sequences preferentially activate non-rodent immune cells. The Py-rich and TG sequences are only minimally immunostimulatory with respect to rodent immune cells, compared to non-rodent immune cells. Thus, it is possible according to the methods of the invention to induce an immune response in a non-rodent subject by administering Py-rich or TG immunostimulatory nucleic acids. The Py-rich and TG immunostimulatory nucleic acids of the invention may optionally include CpG motifs. These findings have important implications for the clinical development of immunostimulatory CpG containing and non-CpG containing nucleic acids.

In one aspect the invention is a pharmaceutical composition comprising an effective amount for stimulating an immune response of isolated Py-rich or TG immunostimulatory nucleic acids, and a pharmaceutically acceptable carrier. In other aspects the invention is a composition of matter, comprising an isolated Py-rich or TG immunostimulatory nucleic acid. In other embodiments, the immunostimulatory nucleic acid may be T-rich. In still other embodiments, the immunostimulatory nucleic acid may be T-rich and also have at least one TG motif.

Preferably the Py-rich nucleic acid is a T-rich nucleic acid. In some embodiments the T-rich immunostimulatory nucleic acid is a poly T nucleic acid comprising 5' TTTT 3'. In yet other embodiments the poly T nucleic acid comprises 5' X₁ X₂ TTTT X₃ X₄ 3' wherein X₁, X₂, X₃ and X₄ are nucleotides. In some embodiments X₁ X₂ is TT and/or X₃ X₄ is TT. In other embodiments X₁ X₂ is selected from the group consisting of TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, CG, GT, GG, GA, and GC; and/or X₃ X₄ is selected from the group consisting of TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, CG, GT, GG, GA, and GC.

The T-rich immunostimulatory nucleic acid may have only a single poly T motif or it may have a plurality of poly T nucleic acid motifs. In some embodiments the T-rich immunostimulatory nucleic acid comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 T motifs. In other embodiments it comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 CpG motifs. In preferred embodiments the plurality of CpG motifs and poly T motifs are interspersed.

In yet other embodiments at least one of the plurality of poly T motifs comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, or at least 9 contiguous T nucleotide residues. In other embodiments the plurality of poly T motifs is at least 3 motifs and wherein at least 3 motifs each comprises at least 3 contiguous T nucleotide residues or the plurality of poly T motifs is at least 4 motifs and wherein the at least 4 motifs each comprises at least 3 contiguous T nucleotide residues.

In some cases the T-rich immunostimulatory nucleic acid may be five of poly T motifs but may rather comprise a nucleotide composition of greater than 25% T. In other embodiments the T-rich immunostimulatory nucleic acids have poly T motifs and also comprise a nucleotide composition of greater than 25% T. In preferred embodiments the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater

than 35% T, greater than 40% T, greater than 50% T, greater than 60% T, greater than 80% T, or greater than 90% T nucleotide residues. In important embodiments, the nucleic acid is at least 50% T.

The T-rich and TG immunostimulatory nucleic acids can have any length greater than 7 nucleotides, but in some embodiments can be between 8 and 100 nucleotides residues in length. In preferred embodiments the T-rich immunostimulatory nucleic acid comprises at least 20 nucleotides, at least 24 nucleotides, at least 27, nucleotides, or at least 30 nucleotides. In preferred embodiments, the TG immunostimulatory nucleic acid is between 15 and 25 nucleotides in length. The T-rich and TG immunostimulatory nucleic acids may be single stranded or double stranded.

In one preferred embodiment, the immunostimulatory nucleic acid has a T-rich region located in the middle of its length (i.e., an approximately equal number of nucleotides flank the T-rich region on the 5' and 3' ends).

The T rich nucleic acid in some embodiments is selected from the group consisting of SEQ ID NO: 59-63, 73-75, 142, 215, 226, 241, 267-269, 282, 301, 304, 330, 342, 358, 370-372, 393, 433, 471, 479, 486, 491, 497, 503, 556-558, 567, 694, 793-794, 797, 833, 852, 861, 867, 868, 882, 886, 905, 907, 908, and 910-913. In other embodiments the T rich nucleic acids are sequences selected from the group consisting of SEQ ID NO: 64, 98, 112, 146, 185, 204, 208, 214, 224, 233, 244, 246, 247, 258, 262, 263, 265, 270-273, 300, 305, 316, 317, 343, 344, 350, 352, 354, 374, 376, 392, 407, 411-413, 429-432, 434, 435, 443, 474, 475, 498-501, 518, 687, 692, 693, 804, 862, 883, 884, 888, 890, and 891.

In other embodiments the Py-rich immunostimulatory nucleic acid is a C-rich nucleic acid. An immunostimulatory C-rich nucleic acid is a nucleic acid including at least one and preferably at least 2 poly-C regions or which includes 50% or greater C nucleotides.

The Py-rich and TG immunostimulatory nucleic acids may include one or more CpG motifs. The motifs may be methylated or unmethylated. In other embodiments the Py-rich and TG immunostimulatory nucleic acids are free of one or more CpG dinucleotides.

In other embodiments the Py-rich and TG immunostimulatory nucleic acids also include poly-A, poly G, and/or poly C motifs. In yet other embodiments the Py-rich or

- 5 -

TG immunostimulatory nucleic acid is free of two poly C sequences of at least 3 contiguous C nucleotide residues or is free of two poly A sequences of at least 3 contiguous A nucleotide residues. In other embodiments the Py-rich or TG immunostimulatory nucleic acid comprises a nucleotide composition of greater than 25% C or greater than 25% A. In yet other embodiments the Py-rich or TG immunostimulatory nucleic acid is free of poly-C sequences, poly-G sequences or poly-A sequences.

A poly G nucleic acid in some embodiments is selected from the group consisting of SEQ ID NO: 5, 6, 73, 215, 267-269, 276, 282, 288, 297-299, 335, 339, 386, 387, 444, 476, 531, 537-539, 733, 768, 795, 796, 914-925, 928-931, 933-936, and 938. In other embodiments the poly G nucleic acid includes a sequence selected from the group consisting of SEQ ID NO: 67, 80-82, 141, 147, 148, 173, 178, 183, 185, 214, 224, 264, 265, 315, 329, 434, 435, 475, 519, 521-524, 526, 527, 535, 554, 565, 609, 628, 660, 661, 662, 725, 767, 825, 856, 857, 876, 882, 909, 926, 927, 932, and 937.

According to another aspect of the invention, the immunostimulatory nucleic acids may be defined as those which possess a TG motif, herein referred to as TG immunostimulatory nucleic acids. The TG nucleic acid in one embodiment contains at least one TG dinucleotide having a sequence including at least the following formula: 5'N₁X₁TX₂N₂3'. In related embodiments, N₁ is a nucleic acid sequence composed of a number of nucleotides ranging from (1-N₁) to (21-N₁) and N₂ is a nucleic acid sequence composed of a number of nucleotides ranging from (1-N₂) to (21-N₂). In a preferred embodiment, X₂ is thymidine.

In other embodiments, the TG nucleic acid has at least the following formula: 5'X₁X₂TX₃X₄3'. In yet another embodiment, the TG nucleic acid comprises the following sequence: 5'N₁X₁X₂TX₃X₄N₂3'. In a related embodiment, N₁ is a nucleic acid sequence composed of a number of nucleotides ranging from (3-N₁) to (19-N₁) and N₂ is a nucleic acid sequence composed of a number of nucleotides ranging from (3-N₂) to (19-N₂). In one preferred embodiment, X₂ is thymidine. X₃X₄ are nucleotides which may be selected from the group consisting of GT, GG, GA, AA, AT, AG, CT, CA, CG, TA and TT, and X₃X₄ are nucleotides which may be selected from the group consisting of TT, CT, AT, AG, CG, TC, AC, CC, TA, AA, and CA. In some preferred embodiments, X₃ is a thymidine. In important embodiments, X₃X₄ are nucleotides

- 6 -

selected from the group consisting of TT, TC, TA and TG. In other embodiments X₃X₄ are GA or GT and X₃X₄ are TT. In yet other embodiments X₃ or X₄ or both are purines and X₃ or X₄ or both are pyrimidines or X₃X₄ are Cpa and X₃ or X₄ or both are pyrimidines. In one embodiment X₃ is a T and X₄ is a pyrimidine.

In one embodiment the 5' X₁X₂TX₃X₄ 3' sequence of the TG nucleic acid or the entire length or some fragment thereof of the TG nucleic acid is a non-palindromic sequence, and in other embodiments it is a palindromic sequence.

In some preferred embodiments, the TG nucleic acid is also T-rich.

The Py-rich and TG immunostimulatory nucleic acids in some embodiments have a nucleotide backbone which includes at least one backbone modification, such as a phosphorothioate modification. The nucleotide backbone may be chlorine, or preferably the nucleotide backbone is entirely modified. In one preferred embodiment, the immunostimulatory nucleic acid has a poly T motif and a phosphorothioate backbone.

In another aspect, the invention is a composition of an immunostimulatory nucleic acid, in the form of a Py-rich or a TG nucleic acid, and an antigen, wherein the nucleic acid is free of unmethylated CpG motifs.

Another composition of the invention is a Py-rich or TG immunostimulatory nucleic acid and an anti-microbial agent, wherein the Py-rich or TG nucleic acid is free of unmethylated CpG motifs. Preferably the anti-microbial agent is selected from the group consisting of an anti-viral agent, an anti-parasitic agent, an anti-bacterial agent and an anti-fungal agent.

A composition of a sustained release device including a Py-rich and/or TG immunostimulatory nucleic acid, wherein the Py-rich and/or TG nucleic acid is free of unmethylated CpG motifs, is provided according to another aspect of the invention.

The invention also includes nutritional supplements of a Py-rich or TG immunostimulatory nucleic acid in a daily device selected from the group consisting of a capsule, a pill, and a sublingual tablet, wherein the Py-rich or TG nucleic acid is free of unmethylated CpG motifs.

It should be understood that when it is useful to administer a Py-rich e.g., poly T, T-rich, C-rich, or TG oligonucleotide and a CpG oligonucleotide, it may also be desirable to co-administer a Py-rich or a TG oligonucleotide together with a physically separate CpG, Py-rich or TG oligonucleotide. Alternatively, the CpG, Py-rich or TG

motif may be present on the same contiguous nucleic acid as the Py-rich or TG oligonucleotide. In yet a further embodiment, all or some combination of Py-rich, TG and CpG nucleic acids may be co-administered either on separate nucleic acids or in same nucleic acid molecule. By co-administer it is intended that the nucleic acids be administered close enough in time to one another to achieve a combined benefit of both oligonucleotides, preferably more than the benefit achieved by administering each of the oligonucleotides alone at the same dose.

CpG oligonucleotides have, in general, the formula $5'X_1X_2CX_3X_43'$, wherein X_1 , X_2 , X_3 and X_4 are nucleotides and wherein at least the C of CpG is unmethylated. Preferred CpG oligonucleotides are 8-100 nucleotides in length and have modified back bones. Particular structures are detailed in the published PCT applications, U.S. applications and references cited herein, the disclosures of which are incorporated herein in their entirety. In one embodiment, the CpG oligonucleotide is free of poly T and TG motifs and is not T-rich.

In other embodiments, the CpG oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 1, 3, 4, 14-16, 18-24, 28, 29, 33-46, 49, 50, 52-56, 58, 64-67, 69, 71, 72, 76-87, 90, 91, 93, 94, 96, 98, 102-124, 126-128, 131-133, 136-141, 146-150, 152-153, 155-171, 173-178, 180-186, 188-198, 201, 203-214, 216-220, 223, 224, 227-240, 242-256, 258, 260-265, 270-273, 275, 277-281, 286-287, 292, 295-296, 300, 302, 305-307, 309-312, 314-317, 320-327, 329, 335, 337-341, 343-352, 354, 357, 361-365, 367-369, 373-376, 378-385, 388-392, 394, 395, 399, 401-404, 406-426, 429-433, 434-437, 439, 441-443, 445, 447, 448, 450, 453-456, 460-464, 466-469, 472-475, 477, 478, 480, 483-485, 488, 489, 492, 493, 495-502, 504-505, 507-509, 511, 513-529, 532-541, 543-555, 564-566, 568-576, 578, 580, 599, 601-605, 607-611, 613-615, 617, 619-622, 625-646, 648-650, 653-664, 666-697, 699-706, 708, 709, 711-716, 718-732, 736, 737, 739-744, 746, 747, 749-761, 763, 766-767, 769, 772-779, 781-783, 785-786, 790-792, 798-799, 804-808, 810, 815, 817, 818, 820-832, 835-846, 849-850, 855-859, 862, 865, 872, 874-877, 879-881, 883-885, 888-904, and 909-913.

In another embodiment, the Py-rich or TG oligonucleotide is free of a CpG motif. This embodiment of the invention also involves pharmaceutical compositions and kits which contain both a CpG oligonucleotide (which can be free of poly T and TG motifs and not be T-rich) and a Py-rich and/or TG oligonucleotide physically separate

from the CpG oligonucleotide. The pharmaceutical preparations are in effective amounts and typically include pharmaceutically acceptable carriers, all as set forth in detail herein with respect to Py-rich and TG oligonucleotides. The kits include at least one container containing an oligonucleotide which is a Py-rich or TG oligonucleotide (or some combination thereof). The same container, or in other embodiments, a second container, may contain an oligonucleotide with a CpG motif, which may be free of Py-rich and/or TG motifs. The kit also contains instructions for administering the oligonucleotides to a subject. The kits also may include a container containing a solvent or a diluent.

In summary, as if fully recited herein, a CpG oligonucleotide physically separate from the Py-rich or TG oligonucleotide can be used together with the Py-rich or TG oligonucleotides in the methods, compositions and products described above.

The invention relates in other aspects to immunostimulatory oligonucleotides which have chimeric backbones and which do not require the presence of a CpG motif. The invention is based in part on the discovery that nucleic acid sequences which did not contain CpG motifs were immunostimulatory, and that those which have chimeric backbones have unexpectedly enhanced immune stimulating properties. Thus the invention in one aspect relates to a composition of an oligonucleotide having a formula: $5'Y_1N_1ZN_2Y_23'$, wherein Y_1 and Y_2 are, independent of one another, nucleic acid molecules having between 1 and 10 nucleotides, wherein Y_1 includes at least one modified internucleotide linkage and Y_2 includes at least one modified internucleotide linkage and wherein N_1 and N_2 are nucleic acid molecules, each independent of one another, having between 0 and 5 nucleotides, but wherein N_1ZN_2 has at least 6 nucleotides in total and wherein the nucleotides of N_1ZN_2 have a phosphodiester backbone, and wherein Z is an immunostimulatory nucleic acid motif but does not include a CG. In one embodiment Z is a nucleic acid sequence selected from the group consisting of TTTT, TG, and a sequence wherein at least 50% of the bases of the sequence are Ts.

In some embodiments Y_1 and/or Y_2 have between 3 and 8 nucleotides. In other embodiments Y_1 and/or Y_2 are comprised of at least three Cs, at least four Cs, least seven Cs, or all Cs. In other embodiments Y_1 and/or Y_2 are selected from the group consisting of TCGTCG, TCGTCGT, and TCGTCGTT (SEQ ID NO:1145). In yet other

embodiments Y_1 and/or Y_2 include at least one, two, three, four, or five poly-A, poly-T, or poly-C sequences.

The center nucleotides (N_1ZN_2) of the formula $Y_1N_1ZN_2Y_2$ have phosphodiester internucleotide linkages and Y_1 and Y_2 have at least one modified internucleotide linkages. In some embodiments Y_1 and/or Y_2 have at least two modified internucleotide linkages. In other embodiments Y_1 and/or Y_2 have between two and five modified internucleotide linkages. In yet other embodiments Y_1 has two modified internucleotide linkages and Y_2 has five modified internucleotide linkages or Y_1 has five modified internucleotide linkages and Y_2 has two modified internucleotide linkages. The modified internucleotide linkage in some embodiments is a phosphorothioate modified linkage, a phosphonodithioate modified linkage or a P-ethoxy modified linkage.

Portions of the formula $Y_1N_1ZN_2Y_2$ may optionally form a palindromic. Thus, in some embodiments the nucleotides of N_1ZN_2 form a palindromic. In some embodiments the palindromic is not a direct repeat. In yet other embodiments the nucleotides of N_1ZN_2 do not form a palindromic.

According to other embodiments N_1ZN_2 has a sequence of nucleotides selected from the group consisting of GATTTTATCGTC (SEQ ID NO:1098),

TCGATTTTTCGA (SEQ ID NO:1099), TCAATTTTATGA (SEQ ID NO:1100),
GTTTITTAACGAC (SEQ ID NO:1101), TCAATTTTITGA (SEQ ID NO:1102),
ACGTTTTTACGT (SEQ ID NO:1103), TCGTTTTTACGA (SEQ ID NO:1104),
TCGATTTTACGTCGA (SEQ ID NO:1105), AATTTTTTAAACGTT (SEQ ID NO:
1106), TCGTTTTTAAACGA (SEQ ID NO:1107), ACGTTTTTAAACGT (SEQ ID
NO:1108), GATTTTATCGTC (SEQ ID NO:1109), GACGATTTTTCGTC (SEQ ID
NO:1110), GATTTTACGTCGTC (SEQ ID NO:1111), GATTTTACGTC (SEQ ID
NO:1112), AATTTATCGT (SEQ ID NO:1113), AACGATTTTTCGTT (SEQ ID NO:
1114), TCACTTTTITGA (SEQ ID NO:1115), TCGTATTTTA (SEQ ID NO:1116),
ACTTTTGTACCGGT (SEQ ID NO:1117), TCGATTTTTCGACGTCGA (SEQ ID
NO:1118), ACGATTTTTCGT (SEQ ID NO:1119), GATGATCGTC (SEQ ID NO:
1120), TCGATGTCGA (SEQ ID NO:1121), TCAATGTCGA (SEQ ID NO:1122),
GTGTTTACGAC (SEQ ID NO:1123), TCAATGTCGA (SEQ ID NO:1124),
ACGTGTAACGT (SEQ ID NO:1125), TCGTGTACGA (SEQ ID NO:1126),
TCGATGTACGTCGA (SEQ ID NO:1127), AATGTTAAACGTT (SEQ ID NO:1128),

TCGTTGTAAACGA (SEQ ID NO:1129), ACGTTTAAACGT (SEQ ID NO:1130),
GATGTATCGTC (SEQ ID NO:1131), GACGATGTGTC (SEQ ID NO:1132),
GATGACGTCGTC (SEQ ID NO:1133), GATGTACGTC (SEQ ID NO:1134),
ATGATCGT (SEQ ID NO:1135), AACGATGTGTC (SEQ ID NO:1136),
TCGACTGTTGA (SEQ ID NO:1137), TCGTATGA (SEQ ID NO:1138),
ACTGTTACCGGT (SEQ ID NO:1139), TCGATGTTCGACGTCGA (SEQ ID NO:
1140), and ACGATGTGTC (SEQ ID NO:1141).

The composition may optionally include a pharmaceutical carrier and/or be formulated in a delivery device. In some embodiments the delivery device is selected from the group consisting of cationic lipids, cell permeating proteins, and sustained release devices. In one preferred embodiment the sustained release device is a biodegradable polymer. In another embodiment the sustained release device is a microparticle.

In another aspect the invention is a composition of an immunostimulatory oligonucleotide having the formula $Y_1N_1ZN_2Y_2$, and an antigen.

Another composition of the invention is an immunostimulatory oligonucleotide having the formula $Y_1N_1ZN_2Y_2$, and an anti-microbial therapeutic agent. Preferably the anti-microbial therapeutic agent is selected from the group consisting of an anti-viral agent, an anti-parasitic agent, an anti-bacterial agent, or an anti-fungal agent.

A composition of a sustained release device including an immunostimulatory oligonucleotide having the formula $Y_1N_1ZN_2Y_2$, is provided according to another aspect of the invention.

The invention also includes nutritional supplements of an immunostimulatory oligonucleotide having the formula $Y_1N_1ZN_2Y_2$, in a delivery device selected from the group consisting of a capsule, a sublingual tablet, and a pill.

In another aspect the compositions described above also include an immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide, or a Py-rich sequence wherein the immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence has a different sequence than the oligonucleotide comprising 3' $Y_1N_1ZN_2Y_2$ 3'.

In some embodiments the immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence has a

completely phosphodiester backbone and in other embodiments the immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence has a modified backbone, which optionally may have internucleotide linkages selected from the group consisting of phosphorothioate, phosphorodithioate, and p-ethoxy.

In one embodiment immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a formula comprising: $5' X_1 X_2 COX_3 X_4 3'$ wherein X_1 , X_2 , X_3 and X_4 are nucleotides. In other embodiments the immunostimulatory nucleic acid sequence includes at least the following formula: $5' TCNTX_1 X_2 COX_3 X_4 3'$ wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides, wherein at least one nucleotide has a modified internucleotide linkage, and wherein the nucleic acid has less than or equal to 100 nucleotides. According to some embodiments $X_1 X_2$ are nucleotides selected from the group consisting of GT, GG, GA and AA and $X_3 X_4$ are nucleotides selected from the group consisting of TT, CT or GT. In a preferred embodiment $X_1 X_2$ are GA and $X_3 X_4$ are TT.

In another embodiment the immunostimulatory nucleic acid sequence having an unmethylated CG dinucleotide includes at least one of the following sequences:

- ATCGACTCTCGAGCGTTTC (SEQ ID No. 15); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 32); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 31);
- ATCGACTCTCGAGCGTTTC (SEQ ID No. 18); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 28); GCGGGG (SEQ ID No. 12); TCCATGACGGTCTCTGATGCT (SEQ ID No. 35); TCCATGCGCGTCTCTGATGCT (SEQ ID No. 34);
- TCCATGACGGTCTCTGATGCT (SEQ ID No. 7); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 38); GCGGTCACTCTCTGACGGG (SEQ ID No. 41);
- TCCATGTCCGTCTCTGATGCT (SEQ ID No. 37); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 36); TCCATGCCGTCTCTGATGCT (SEQ ID No. 33);
- TCCATGACGGTCTCTGATGCT (SEQ ID No. 3); TCCATGACGGTCTCTGATGCT (SEQ ID No. 7); TCCATGACGGTCTCTGATGCT (SEQ ID No. 39);
- TCCATGACGGTCTCTGATGCT (SEQ ID No. 48); TCCATGACGGTCTCTGATGCT (SEQ ID No. 10); ATGACGTCTCTGACGGT (SEQ ID No. 70);
- TCTCCACGCGCGCCAT (SEQ ID No. 72); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 73); TCCATGCGGTCTCTGATGCT (SEQ ID No. 74);

TCCATGACGGTCTCTGATGCT (SEQ ID No. 76); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 77); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 52);

TCCATGTCCGTCTCTGATGCT (SEQ ID No. 121); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 208); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 120);

TCCATGTCCGTCTCTGATGCT (SEQ ID No. 81); TCCATGACGGTCTCTGATGCT (SEQ ID No. 82); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 47);

TCCATGTCCGTCTCTGATGCT (SEQ ID No. 46);

TCCATGTCCGTCTCTGATGCT (SEQ ID No. 49);

GCATGACGGTCTCTGATGCT (SEQ ID No. 56); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 48); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 84);

TCCATGTCCGTCTCTGATGCT (SEQ ID No. 50); TCCATGTCCGTCTCTGATGCT (SEQ ID No. 51); and TCCATGTCCGTCTCTGATGCT (SEQ ID No. 85). In another embodiment the

immunostimulatory nucleic acid having a Py-rich or TG sequence is a nucleic acid as described above.

In another aspect the invention relates to pharmaceutical compositions and kits which contain both an oligonucleotide having the formula $Y_1 N_1 Z N_2 Y_2$ and a CpG oligonucleotide (which optionally may be free of poly T and TG motifs and not be Py-rich), a Py-rich and/or TG oligonucleotide physically separate from the oligonucleotide having the formula $Y_1 N_1 Z N_2 Y_2$. The pharmaceutical preparations are in effective

amounts and typically include pharmaceutically acceptable carriers, all as set forth in detail herein. The kits include at least one container containing an oligonucleotide having the formula $Y_1 N_1 Z N_2 Y_2$. The same container, or in other embodiments, a second container, may contain an oligonucleotide with a CpG motif, which optionally may be free of Py-rich and/or TG motifs and/or a Py-rich or TG oligonucleotide (or some combination thereof). The kit also contains instructions for administering the oligonucleotides to a subject. The kits also may include a container containing a solvent or a diluent.

In summary, as is fully recited herein, an oligonucleotide having the formula $Y_1 N_1 Z N_2 Y_2$, which is physically separate from the CpG, Py-rich or TG oligonucleotide can be used together with the CpG, Py-rich, TG oligonucleotide, in the methods, compositions and products described herein.

- 13 -

In another aspect the invention relates to a pharmaceutical composition including at least two oligonucleotides of the invention, wherein the at least two oligonucleotides have different sequences from one another and a pharmaceutically acceptable carrier.

A vaccine formulation is provided according to another aspect of the invention.

5 The vaccine includes any of the compositions of the invention in combination with an antigen.

According to another aspect of the invention a method of stimulating an immune response is provided. The method involves administering a Py-rich or a TG

10 immunostimulatory nucleic acid to a non-todent subject in an amount effective to induce an immune response in the non-todent subject. Preferably the Py-rich or TG immunostimulatory nucleic acid is administered orally, locally, in a sustained release device, mucosally to a mucosal surface, systemically, parenterally, or intramuscularly.

When the Py-rich or TG immunostimulatory nucleic acid is administered to the mucosal surface it may be delivered in an amount effective for inducing a mucosal immune

15 response or a systemic immune response. In preferred embodiments the mucosal surface is selected from the group consisting of an oral, nasal, rectal, vaginal, and ocular surface.

In some embodiments the method includes exposing the subject to an antigen wherein the immune response is an antigen-specific immune response. The antigen may be encoded by a nucleic acid vector which can be delivered to the subject. In some 20 embodiments the antigen is selected from the group consisting of a tumor antigen, a viral antigen, a bacterial antigen, a parasitic antigen and a peptide antigen.

Py-rich and TG immunostimulatory nucleic acids are capable of providing a broad spectrum of immune response. For instance these immunostimulatory nucleic acids can be used to redirect a Th2 to a Th1 immune response. Py-rich and TG nucleic acids may also be used to activate an immune cell, such as a leukocyte, a dendritic cell, 25 and an NK cell. The activation can be performed *in vivo*, *in vitro*, or *ex vivo*, i.e., by isolating an immune cell from the subject, contacting the immune cell with an effective amount to activate the immune cell of the Py-rich or TG immunostimulatory nucleic acid and re-administering the activated immune cell to the subject. In some embodiments the dendritic cell expresses a cancer antigen. The dendritic cell can be exposed to the cancer antigen *ex vivo*. 30

- 14 -

The immune response produced by Py-rich or TG nucleic acids may also result in induction of cytokine production, e.g., production of IL-6, IL-12, IL-18 TNF, IFN- α and IFN- γ .

5 In still another embodiment, the Py-rich and TG nucleic acids are useful for treating cancer. The Py-rich and TG nucleic acids are also useful according to other aspects of the invention in preventing cancer (e.g., reducing a risk of developing cancer) in a subject at risk of developing a cancer. The cancer may be selected from the group

10 consisting of biliary tract cancer, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, gastric cancer, intraepithelial neoplasia, lymphoma, liver cancer, lung cancer (e.g. small cell and non-small cell), melanoma, neuroblastomas, oral cancer, ovarian cancer, pancreas cancer, prostate cancer, rectal cancer, sarcoma, thyroid cancer, and renal cancer, as well as other carcinomas and sarcomas. In some important embodiments, the cancer is selected from the group consisting of bone cancer, brain and CNS cancer, connective tissue cancer, esophageal cancer, eye cancer, Hodgkin's lymphoma, larynx cancer, oral cavity cancer, skin cancer, and testicular cancer.

15 Py-rich and TG nucleic acids may also be used for increasing the responsiveness of a cancer cell to a cancer therapy (e.g., an anti-cancer therapy), optionally when the Py-rich or TG immunostimulatory nucleic acid is administered in conjunction with an anti-cancer therapy. The anti-cancer therapy may be a chemotherapy, a vaccine (e.g., an *in vitro* primed dendritic cell vaccine or a cancer antigen vaccine) or an antibody based therapy. This latter therapy may also involve administering an antibody specific for a cell surface antigen of, for example, a cancer cell, wherein the immune response results in antigen dependent cellular cytotoxicity (ADCC). In one embodiment, the antibody 20 may be selected from the group consisting of Rituximab, Herceptin, Quindramet, Panorex, IDBC-Y238, BEC2, C223, Onasolym, SMART M193, ATRAGEN, Ovaren, Becare, LDP-03, *loc* 96, MDX-210, MDX-11, MDX-22, OVI03, 362W94, anti-VEGF, Zampax, MDX-220, MDX-447, MELIMADUNE-2, MELIMADUNE-1, CEACDIB, Prexagen, NovoMAB-Q2, TNF, Glucanab-H, GNI-250, BMD-72000, LymphoCide, CMA 676, Monopharm-C, 4B5, *loc* egf-6, *loc* c5, BABS, anti-FLK-2, MDX-260, ANA AB, SMART ID10 Ab, SMART ABL 364 Ab and ImmunALT-CEA. 30

Thus, according to some aspects of the invention, a subject having cancer or at risk of having a cancer is administered an immunostimulatory nucleic acid and an anti-

cancer therapy. In some embodiments, the anti-cancer therapy is selected from the group consisting of a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine. The chemotherapeutic agent may be selected from the group consisting of methotrexate, vincristine, adriamycin, cisplatin, non-sugar containing

- 5 chloroethyl/nitrosourea, 5-fluorouracil, mitomycin C, bleomycin, doxorubicin, decarbazine, taxol, flutamide, Meglumine GLA, valrubicin, carmustine and polifeposum, MM0270, BAY 12-9366, RAS farnesyl transferase inhibitor, farnesyl transferase inhibitor, MMP, MTAL/Y231514, LY2646161/omectazol, Glumolec, CI-994, TNF-470, Hyacinth/Troponeum, PKC412, Valopodar/PSC833,
- 10 Novotrans/Mitoxantrene, Metreux/Suramin, Bafinastat, E7070, BCH-4556, CS-682, 9-AC, AG3340, AG3433, Inocel/VX-710, VX-853, ZD0101, ISI641, ODN 698, TA 2516/Marmistat, BB2516/Marmistat, CDP 845, D2163, PD183805, DX8951f, Lemonal DP 2202, FK 317, Pielbani/OK-432, AD 32/Vulubicin, Menastren/dramium derivative, Temodal/Temozolomide, Evance/liposomal doxorubicin, Yewtuxan/Paclitaxel,
- 15 Taxol/Paclitaxel, Xeloda/Capecitabine, Furtulow/Doxifluridine, Cyclopaax/oral paclitaxel, Oral Taxoid, SFU-077/Cisplatin, HMR 1275/Flavopiridol, CP-358 (774)EGFR, CP-609 (754)/RAS oncogene inhibitor, BMS-182751/oral platinum, UFT(Tegafur/Uracil), Erganisoa/Levamisole, Eaulumcel/776C85/5FU enhancer, Camptox/Levamisole, Compoxar/Iribotocan, Tumodex/Ralireved, Leustatin/Cladribine, Poxez/Paclitaxel, Doxil/liposomal doxorubicin, Caelys/liposomal doxorubicin, Fludam/Fludamine, Pharmarubicin/Epirubicin, DepoCyt, ZD1839, LU 79553/Bis-Naphthalimide, LU 103793/Dolanatin, Caelys/liposomal doxorubicin, Genazax/Gemcitabine, ZD 0473/Axommed, YM 116, Iodine seeds, CDK4 and CDK2 inhibitors, PARP inhibitors,
- 20 D4809/Dactinosamide, Itra/Mesenz/Isoamide, Vunior/Teniposide, ZD 9331, Pamplatin/Carboplatin, Platinol/cisplatin, Vepeside/Etoposide, ZD 9331, Taxotere/Doxetaxel, prodrug of gemtine arabinoside, Taxane Analog, nitrosourea, alkylating agents such as melphalan and cyclophosphamide, Aminoglutethimide, Aspergillase, Busulfan, Carboplatin, Chlorambucil, Cytarabine HCl, Daclizumycin, Daunorubicin HCl, Benamurine phosphate sodium, Etoposide (VP16-213), Flouxuridine,
- 30 Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxyureamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna,

Mitomine (6-p-DDD), Mitoxantrene HCl, Oestrone, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thiotepa, Vinblastine sulfate, Anastroline (m-AMSA), Anastroline, Eribropietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguanone (methy/-GAG; methyl glyoxal bis-guanyldrazones; MGBG), Pentostatin (2'-deoxycobfomycin), Semustine (methy/-CCNU), Teniposide (VM-26) and Vinorelbine sulfate, but it is not so limited.

The immunotherapeutic agent may be selected from the group consisting of Ributaxin, Herceptin, Quaramet, Penorex, IDEC-Y2B8, BEC3, C225, Oncohyt, SMART M195, ATRAQEN, Ovarax, Bexser, LDP-03, Ior 16, MDX-210, MDX-11, MDX-22, OV103, 362ZW94, anti-VBGF, Zenasex, MDX-220, MDX-447,

10 MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Presteg4, Novemab-G2, TNT, Gliomab-H, QNU-250, EMD-72000, LymphoCide, CMA 676, Manopharm-C, 4B5, Ior egl-3, Ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART ID10 Ab, SMART ABL 364 Ab and ImmunRAIT-CEA, but it is not so limited.

15 The cancer vaccine may be selected from the group consisting of EGF, Anti-idiotypic cancer vaccines, Op75 antigen, GMK melanoma vaccine, MOV ganglioside conjugate vaccine, Hec2/here, Ovarax, M-Vax, O-Vax, L-Vax, STb-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, psipido antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BC3 vaccine, TA-HPV, TA-CIN, DISC-virus and ImmunCys/TheraCys, but it is not so limited.

20 In still another embodiment of the methods directed to preventing or treating cancer, the subject may be further administered interferon- α .

The invention in other aspects relates to methods for preventing disease in a subject. The method involves administering to the subject a Py-dich or a TG immunostimulatory nucleic acid on a regular basis to promote immune system responsiveness to prevent disease in the subject. Examples of diseases or conditions sought to be prevented using the prophylactic methods of the invention include microbial infections (e.g., sexually transmitted diseases) and asphyliotic shock from food allergies.

30 In other aspects, the invention is a method for inducing an innate immune response by administering to the subject a Py-dich or a TG immunostimulatory nucleic acid in an amount effective for activating an innate immune response.

- 17 -

According to another aspect of the invention a method for treating or preventing a viral or retroviral infection is provided. The method involves administering to a subject having or at risk of having a viral or retroviral infection, an effective amount for treating or preventing the viral or retroviral infection of any of the compositions of the invention. In some embodiments the virus is caused by a hepatitis virus, HIV, hepatitis B, hepatitis C, herpes virus, or papillomavirus.

A method for treating or preventing a bacterial infection is provided according to another aspect of the invention. The method involves administering to a subject having or at risk of having a bacterial infection, an effective amount for treating or preventing the bacterial infection of any of the compositions of the invention. In one embodiment the bacterial infection is due to an intracellular bacteria.

In another aspect the invention is a method for treating or preventing a parasitic infection by administering to a subject having or at risk of having a parasitic infection, an effective amount for treating or preventing the parasitic infection of any of the compositions of the invention. In one embodiment the parasitic infection is due to an intracellular parasite. In another embodiment the parasitic infection is due to a non-intracellular parasite.

In some embodiments the subject is a human and in other embodiments the subject is a non-human vertebrate selected from the group consisting of a dog, cat, horse, cow, pig, goat, fish, monkey, chicken, and sheep.

In yet another aspect, the invention is a method for treating or preventing asthma, by administering to a subject having or at risk of having asthma, an effective amount for treating or preventing the asthma of any of the compositions of the invention. In one embodiment the asthma is allergic asthma.

In another aspect the invention relates to a method for treating or preventing allergy. The method involves administering to a subject having or at risk of having allergy, an effective amount for treating or preventing the allergy of any of the compositions of the invention.

A method for treating or preventing an immune deficiency is provided according to another aspect of the invention. The method involves administering to a subject having or at risk of an immune deficiency, an effective amount for treating or preventing the immune deficiency of any of the compositions of the invention.

- 18 -

In another aspect the invention relates to a method for inducing a TH1 immune response by administering to a subject any of the compositions of the invention in an effective amount to produce a TH1 immune response.

In one embodiment the methods of the invention involve administering an oligonucleotide of formula $5' \text{Y(N)}_2\text{N}_2\text{Y}_3 3'$ and an immunostimulatory nucleic acid having an unmethylated CG dinucleotide a TG dinucleotide or a T-rich sequence. In an embodiment the oligonucleotide comprising $5' \text{Y(N)}_2\text{N}_2\text{Y}_3 3'$ is administered separately from the immunostimulatory nucleic acid. In some embodiments the oligonucleotide comprising $5' \text{Y(N)}_2\text{N}_2\text{Y}_3 3'$ and the immunostimulatory nucleic acid are administered on an alternating weekly schedule and in other embodiments the oligonucleotide comprising $5' \text{Y(N)}_2\text{N}_2\text{Y}_3 3'$ and the immunostimulatory nucleic acid are administered on an alternating biweekly schedule.

The invention provides in another aspect a composition, comprising an immunostimulatory nucleic acid and an anti-cancer therapy, formulated in a pharmaceutically-acceptable carrier and in an effective amount to treat a cancer or to reduce the risk of developing a cancer. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid.

The invention further provides a kit comprising a first container housing an immunostimulatory nucleic acid and at least one other container (e.g., a second container) housing an anti-cancer therapy, and instructions for use. In one embodiment, the kit further comprises interferon- α , which may be separately housed in yet another container (e.g., a third container). In an important embodiment, the kit comprises a sustained-release vehicle combining an immunostimulatory nucleic acid, and at least one container housing an anti-cancer therapy, and instructions for timing of administration of the anti-cancer therapy. The immunostimulatory nucleic acid may be selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid, wherein the C-rich nucleic acid has a nucleotide sequence comprising SEQ ID NO: 246.

The invention further provides a method for preventing or treating asthma or allergy, comprising administering an immunostimulatory nucleic acid and an asthma/allergy medicament in an effective amount to treat or prevent the asthma or

allergy. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid.

In one embodiment the immunostimulatory nucleic acid is a T-rich nucleic acid.

In a related embodiment, the T-rich nucleic acid has a nucleotide sequence selected from the group consisting of SEQ ID NO: 59-63, 73-75, 142, 215, 226, 241, 267-269, 282, 301, 304, 330, 342, 358, 370-372, 393, 433, 471, 479, 486, 491, 497, 503, 556-558, 567, 694, 793-794, 797, 833, 852, 861, 867, 868, 882, 886, 905, 907, 908, and 910-913. In other embodiments the T-rich nucleic acids are sequence selected from the group

consisting of SEQ ID NO: 64, 98, 112, 146, 185, 204, 208, 214, 224, 233, 244, 246, 247, 258, 262, 263, 265, 270-273, 300, 305, 316, 317, 343, 344, 350, 352, 354, 374, 376, 392, 407, 411-413, 429-432, 434, 435, 443, 474, 475, 498-501, 518, 687, 692, 693, 804, 862, 883, 884, 888, 890, and 891.

In yet a further related embodiment, the T-rich nucleic acid is not a TG nucleic acid. In yet still another embodiment, the T-rich nucleic acid is not a CpG nucleic acid.

In one embodiment, the immunostimulatory nucleic acid is a TG nucleic acid. In a further related embodiment, the TG nucleic acid is not a T-rich nucleic acid. In another related embodiment, the TG nucleic acid is not a CpG nucleic acid.

In one embodiment, the immunostimulatory nucleic acid is a CpG nucleic acid, wherein the CpG nucleic acid has a nucleotide sequence comprising SEQ ID NO: 246.

In another embodiment, the asthma/allergy medicament is a medicament selected from the group consisting of PDE-4 inhibitor, Bronchodilator/beta-2 agonist, K⁺ channel opener, VLA-4 antagonist, Neurexin antagonist, TXA₂ synthesis inhibitor, Xanthine, Arachidonic acid antagonist, 5 lipooxygenase inhibitor, Thromboxan A₂ receptor antagonist, Thromboxane A₂ antagonist, Inhibitor of 5-lipoxygenase activation protein, and Protease inhibitor, but is not so limited. In some important embodiments, the asthma/allergy medicament is a Bronchodilator/beta-2 agonist selected from the group consisting of salmeterol, albuterol, terbutaline, D15722/formoterol, fenoterol, and oriprenaline.

In another embodiment, the asthma/allergy medicament is a medicament selected from the group consisting of Anti-histamines and Prostaglandin inducers. In one embodiment, the anti-histamines is selected from the group consisting of loratadine, cetirizine, buclizine, ceterizine analogues, fexofenadine, terfenadine, desloratadine,

nonsteroidal, epinastine, ebastine, ebastine, astemizole, levocabastine, azelastine, triamterol, terfenadine, mizolastine, beclastine, CS 560, and HSR 609. In another embodiment, the Prostaglandin inducer is S-5751.

In yet another embodiment, the asthma/allergy medicament is selected from the group consisting of Steroids and Immunomodulators. The immunomodulators may be selected from the group consisting of anti-inflammatory agents, leukotriene antagonists, IL4 mutins, Soluble IL-4 receptors, Immunosuppressants, anti-IL-4 antibodies, IL-4 antagonists, anti-IL-5 antibodies, soluble IL-13 receptor-Fe fusion proteins, anti-IL-9 antibodies, CCR3 antagonists, CCR5 antagonists, VLA-4 inhibitors, and Downregulators of IgE, but are not so limited. In one embodiment, the downregulator of IgE is an anti-IgE.

In another embodiment, the Steroid is selected from the group consisting of beclomethasone, fluticasone, triamcinolone, budesonide, and budesonide. In still a further embodiment, the Immunosuppressant is a Tolerizing peptide vaccine.

In one embodiment, the immunostimulatory nucleic acid is administered concurrently with the asthma/allergy medicament. In another embodiment, the subject is an immunocompromised subject.

The immunostimulatory nucleic acids to be administered to a subject in the methods disclosed herein relating to the prevention and treatment of asthma/allergy are as described for other method aspects of the invention.

In another aspect, the invention provides a kit comprising a first container housing an immunostimulatory nucleic acid, and at least another container (e.g., a second container) housing an asthma/allergy medicament, and instructions for use. The immunostimulatory nucleic acid useful in the kit is as described herein. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid. In another important embodiment, the kit comprises a sustained-release vehicle containing an immunostimulatory nucleic acid, and at least one container housing an asthma/allergy medicament, and instructions for timing of administration of the asthma/allergy medicament. The asthma/allergy medicament may be selected from the group of asthma/allergy medicaments described in the foregoing methods directed towards the prevention or treatment of asthma/allergy.

In yet another aspect, the invention provides a composition, comprising an immunostimulatory nucleic acid and an asthma/allergy medicament, formulated in a pharmaceutically-acceptable carrier and in an effective amount for preventing or treating an immune response associated with exposure to a mediator of asthma or allergy. The immunostimulatory nucleic acid may be selected from the group of immunostimulatory nucleic acids described for the foregoing methods and compositions. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid. The asthma/allergy medicament may be selected from the group consisting of asthma medicaments and allergy medicaments as described in the foregoing methods and compositions.

In still a further aspect, the invention provides a composition comprising an immunostimulatory nucleic acid selected from the group consisting of SEQ ID NO: 95-136, SEQ ID NO: 138-152, SEQ ID NO: 154-222, SEQ ID NO: 224-245, SEQ ID NO: 247-261, SEQ ID NO: 263-299, SEQ ID NO: 301, SEQ ID NO: 303-4109, SEQ ID NO: 414-420, SEQ ID NO: 424, SEQ ID NO: 426-947, SEQ ID NO: 959-1022, SEQ ID NO: 1024-1093, and a pharmaceutically acceptable carrier. Preferably the immunostimulatory nucleic acid is present in the composition in an effective amount. In one embodiment, the immunostimulatory nucleic acid is present in an effective amount to induce an immune response. In another embodiment, the immunostimulatory nucleic acid is present in an effective amount to prevent or treat cancer. In yet a further embodiment, the immunostimulatory nucleic acid is present in an effective amount to prevent or treat asthma/allergy. The invention also provides kits comprising any of the foregoing immunostimulatory nucleic acid compositions, and instructions for use.

In another aspect the invention includes a composition of an immunostimulatory nucleic acid consisting essentially of: 5' M₁TCGTCGTTM₂ 3' wherein at least one of the Cs is unmodified, wherein M₁ is a nucleic acid having at least one nucleotide, wherein M₂ is a nucleic acid having between 0 and 50 nucleotides, and wherein the immunostimulatory nucleic acid has less than 100 nucleotides.

In yet other aspects the invention relates to a pharmaceutical composition of an immunostimulatory nucleic acid comprising: 5' TCGTCGTT 3' wherein at least one of the Cs is unmodified, wherein the immunostimulatory nucleic acid has less than 100 nucleotides and a phosphodiester backbone, and a sustained release device. In some

embodiments the sustained release device is a microparticle. In other embodiments the composition includes an antigen.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a histogram of the expression of CD86 (Y-axis) by CD19+ cells following exposure of these cells to the oligonucleotides shown on the X-axis at a concentration of 0.15 µg/ml.

Figure 1B is a histogram of the expression of CD86 (Y-axis) by CD19+ cells following exposure of these cells to the oligonucleotides shown on the X-axis at a concentration of 0.30 µg/ml.

Figure 2 is a graph comparing the abilities of ODN 2137, ODN 2177, ODN 2200 and ODN 2202 to stimulate B cell proliferation at concentrations ranging from 0.2 µg/ml to 20 µg/ml.

Figure 3 is a graph comparing the abilities of ODN 2188, ODN 2189, ODN 2190 and ODN 2182 to stimulate B cell proliferation at concentrations ranging from 0.2 µg/ml to 20 µg/ml.

Fig. 4 is a bar graph depicting dose-dependent B cell activation induced by non-CpG ODN. PBMC of a blood donor were incubated with the indicated concentrations of ODNs 2006 (SEQ ID NO: 246), 2117 (SEQ ID NO: 358), 2137 (SEQ ID NO: 886), 5126 (SEQ ID NO: 1058) and 5162 (SEQ ID NO: 1094) and stained with mAb for CD19 (B cell marker) and CD86 (B cell activation marker, B7-2). Expression was measured by flow cytometry.

Fig. 5 is a bar graph depicting stimulation of B cells by a diverse set of non-CpG ODNs. PBMC of one representative donor were stimulated by 0.4 µg/ml, 1.0 µg/ml or 10.0 µg/ml of the following ODNs: 2006 (SEQ ID NO: 246), 2196 (SEQ ID NO: 913), 2194 (SEQ ID NO: 911), 5162 (SEQ ID NO: 1094), 5163 (SEQ ID NO: 1095), 5168 (SEQ ID NO: 1096) and 5169 (SEQ ID NO: 1097) and expression of the activation marker CD86 (B7-2) on CD19-positive B cells was measured by flow cytometry.

Fig 6 is a bar graph depicting B cell activation by non-CpG ODNs 1982 and 2041. PBMC were incubated with the indicated concentrations of ODN 2006 (SEQ ID NO.: 246), 1982 (SEQ ID NO.: 225) and 2041 (SEQ ID NO.: 282) and B cell activation (expression of the activation marker CD86) was measured by flow cytometry.

Fig. 7 is a bar graph depicting NK cells are activated by non-CpG ODNs. PBMC were incubated with 6 μ g/ml of the following ODNs: 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058) and stained with mAb for CD3 (T cell marker), CD56 (NK cell marker) and CD69 (early activation marker). Expression of CD69 on CD56-positive NK cells was measured by flow cytometry.

Fig. 8 is a bar graph depicting NK-mediated cytotoxicity is enhanced by non-CpG ODN. NK-mediated lysis of K-562 target cells was measured after overnight incubation of PBMC with 6 μ g/ml of the ODN 2006 (SEQ ID NO.: 246), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058).

Fig. 9 is a bar graph depicting NKT cells can be activated by non-CpG ODN. PBMC of one representative donor were incubated with 6 μ g/ml ODN 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058) for 24h and activation of NKT cells was measured by flow cytometry after staining of cells with mAb for CD3 (T cell marker), CD56 (NK cell marker) and CD69 (early activation marker).

Fig. 10 is a bar graph depicting stimulation of monocytes by different CpG and non-CpG ODN. PBMC were incubated with 6 μ g/ml 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2178 (SEQ ID NO.: 428), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911), 5126 (SEQ ID NO.: 1058) and 5163 (SEQ ID NO.: 1095) and stained for CD14 (monocyte marker) and CD80 (B7-1, activation marker). Expression was measured by flow cytometry.

Fig. 11 is a bar graph depicting release of TNF α upon culture of human cells with non-CpG ODN. PBMC were cultured for 24h with or without 6 μ g/ml of the indicated ODNs or 1 μ g/ml LPS as positive control and TNF α measured by ELISA.

Fig. 12 is a bar graph depicting release of IL-6 after culture with non-CpG ODNs shows the same pattern as for TNF α . PBMC were cultured with the indicated ODNs (1.0 μ g/ml) and IL-6 was measured in the supernatants by ELISA.

DETAILED DESCRIPTION

The invention in one aspect involves the finding that pyrimidine (Py) rich and preferably thymidine (T) rich nucleic acids as well as nucleic acids that contain TG dinucleotide motifs are effective in mediating immune stimulatory effects. It was known

in the prior art that CpG containing nucleic acids are therapeutic and prophylactic compositions that stimulate the immune system to treat cancer, infectious diseases, allergy, asthma and other disorders and to help protect against opportunistic infections following cancer chemotherapies. The strong yet balanced, cellular and humoral immune responses that result from CpG stimulation reflect the body's own natural defense system against invading pathogens and cancerous cells. CpG sequences, while relatively rare in human DNA are commonly found in the DNA of infectious organisms such as bacteria. The human immune system has apparently evolved to recognize CpG sequences as an early warning sign of infection, and to initiate an immediate and powerful immune response against invading pathogens without causing adverse reactions frequently seen with other immune stimulatory agents. Thus CpG containing nucleic acids, relying on this innate immune defense mechanism, can utilize a unique and natural pathway for immune therapy. The effects of CpG nucleic acids on immune modulation were discovered by the inventor of the instant patent application and have been described extensively in co-pending patent applications, such as U.S. Patent Application Serial Nos: 08/386,063 filed on 02/07/95 (and related PCT US95/01570); 08/738,652 filed on 10/30/96; 08/960,774 filed on 10/30/97 (and related PCT/US97/19791, WO 98/18810); 09/191,170 filed on 11/13/98; 09/030,701 filed on 02/25/98 (and related PCT/US98/03678; 09/082,649 filed on 05/20/98 (and related PCT/US98/10409); 09/325,193 filed on 06/03/99 (and related PCT/US98/04703); 09/286,098 filed on 04/02/99 (and related PCT/US99/07335); 09/306,281 filed on 05/06/99 (and related PCT/US99/09863). The entire contents of each of these patents and patent applications is hereby incorporated by reference.

The findings of the instant invention are applicable to all of the above described uses of CpG containing nucleic acids as well as any other known use for CpG nucleic acids. The invention involves, in one aspect, the discovery that Py-rich and preferably T-rich and TG nucleic acids have similar immune stimulatory properties to CpG oligonucleotides regardless of whether a CpG motif is present. Thus the invention is

useful for any method for stimulating the immune system using Py-rich or TG nucleic acids. It was also discovered surprisingly according to the invention that oligonucleotides which lack a CpG motif are immune stimulatory and have many of the same prophylactic and therapeutic activities as a CpG oligonucleotide.

A Py-rich nucleic acid is a T-rich or C-rich immunostimulatory nucleic acid. In some embodiments T-rich nucleic acids are preferred. A T-rich nucleic acid is a nucleic acid which includes at least one poly T sequence and/or which has a nucleotide composition of greater than 25% T nucleotide residues. A nucleic acid having a poly-T sequence includes at least four Ts in a row, such as 5'TTTT3'. Preferably the T-rich nucleic acid includes more than one poly T sequence. In preferred embodiments the T-rich nucleic acid may have 2, 3, 4, or more poly T sequences, such as oligonucleotide #2006 (SEQ ID NO:240). One of the most highly immunostimulatory T-rich oligonucleotides discovered according to the invention is a nucleic acid composed entirely of T nucleotide residues, e.g., oligonucleotide #2183 (SEQ ID NO:433). Other T-rich nucleic acids according to the invention have a nucleotide composition of greater than 25% T nucleotide residues, but do not necessarily include a poly T sequence. In these T-rich nucleic acids the T nucleotide residues may be separated from one another by other types of nucleotide residues, i.e., G, C, and A. In some embodiments the T-rich nucleic acids have a nucleotide composition of greater than 35%, 40%, 50%, 60%, 70%, 80%, 90%, and 99% T nucleotide residues and every integer % in between. Preferably the T-rich nucleic acids have at least one poly T sequence and a nucleotide composition of greater than 25% T nucleotide residues.

It was discovered according to the invention that the T content of an ODN has a dramatic effect on the immune stimulatory effect of the ODN and that T-rich ODN can activate multiple human immune cell types in the absence of any CpG motifs. An oligonucleotide having a 3' poly-T region and 2.5 CGs e.g., ODN 2181 (SEQ ID NO:431) is highly immune stimulatory. An oligonucleotide of similar length, ODN 2116 (SEQ ID NO:357) which contains two CG dinucleotides at the 5' end and a poly-C region at the 3' end was also immune stimulatory but to a lesser extent than the T-rich oligonucleotide using standard experimental conditions. Thus, although C and T have almost identical structures, their effects on the immune properties of an ODN are varied. They both are capable of inducing an immune response but to different extents. Thus

both T-rich and C-rich oligonucleotides are useful according to the invention, but T-rich oligonucleotides are preferred. Furthermore, if the T content of the ODN is reduced by incorporating other bases such as G, A, or C, then the immune stimulatory effects are reduced (ODN #2188 (SEQ ID NO:305), 2190 (SEQ ID NO:307), 2191 (SEQ ID NO:308), and 2193 (SEQ ID NO:310)).

A C-rich nucleic acid is a nucleic acid molecule having at least one or preferably at least two poly-C regions or which is composed of at least 50% C nucleotides. A poly-C region is at least four C residues in a row. Thus a poly-C region is encompassed by the formula 5'CCCC3'. In some embodiments it is preferred that the poly-C region have the formula 5'CCCCCCCC3'. Other C-rich nucleic acids according to the invention have a nucleotide composition of greater than 50% C nucleotide residues, but do not necessarily include a poly C sequence. In these C-rich nucleic acids the C nucleotide residues may be separated from one another by other types of nucleotide residues, i.e., G, T, and A. In some embodiments the C-rich nucleic acids have a nucleotide composition of greater than 60%, 70%, 80%, 90%, and 99% C nucleotide residues and every integer % in between. Preferably the C-rich nucleic acids have at least one poly C sequence and a nucleotide composition of greater than 50% C nucleotide residues, and in some embodiments are also T-rich.

As shown in the Examples, several ODNs previously believed to be non-immunostimulatory, including two ODNs SEQ ID NO: 225 and SEQ ID NO: 282 previously described to be non-stimulatory and mainly used as control ODNs (Takahashi, T et al 2000, *J. Immunol.* 164:4458) were found to be immunostimulatory. Our experiments demonstrated that these ODNs can stimulate B cells, although at higher concentrations compared to CpG ODNs (Fig. 6). A long Poly T ODN (30mer) induced, at least in some experiments, comparable strong activation of B cells to one of the strongest CpG ODN activators of B cells. These experiments also revealed the surprising finding that even Poly C ODNs can lead to stimulation of B cells.

Immunostimulation by these ODNs, however, was not limited to human B cells. Differential experimental assays clearly demonstrated in addition that monocytes, NK cells and even NKT cells can be activated by such non-CpG ODNs (Fig. 7-10). In contrast to Poly T and Poly C sequences, immunostimulation by Poly A sequences (at least for monocytes, B and NK cells) was not achieved. Interestingly it was found that the

introduction of a CpG motif into SEQ ID NO.: 225 enhanced the immunostimulatory activity whereas the elongation with a Poly T stretch did not enhance immunostimulation. This suggests that CpG and T-rich ODN may operate through different mechanisms or pathways. It is also possible that insertion of a poly-T motif into a different position of SEQ ID NO.: 225 may result in a change in immunostimulatory properties.

A "TG nucleic acid" or a "TG immunostimulatory nucleic acid" as used herein is a nucleic acid containing at least one TpG dinucleotide (thymidine-guanine dinucleotide sequence, i.e., "TG DNA" or DNA containing a 5' thymidine followed by 3' guanosine and linked by a phosphate bond) and activates a component of the immune system.

In one embodiment the invention provides a TG nucleic acid represented by at least the formula:



wherein X_1 and X_2 are nucleotides and N is any nucleotide and N_1 and N_3 are nucleic acid sequences composed of any number of N provided that the sum total of N_1 and N_3 is in the range of 11 to 21. As an example, if N_1 is 5, then N_3 may be 6 (leading to a total length for the oligonucleotide of 15 nucleotides). The TG may be located anywhere within the oligonucleotide stretch, including the 5' end, the center and the 3' end. Thus, N_1 may be zero through to 21, inclusive, provided that N_3 is appropriately chosen to give a sum of N_2 and N_1 equal to 11 through to 21, inclusive. Similarly, N_2 may be zero through to 21, inclusive, provided that the sum total of N_1 and N_2 equals 11 to 21, inclusive. In some embodiments X_1 is adenine, guanine, or thymidine and X_2 is cytosine, adenine, or thymidine. In one preferred embodiment, X_2 is thymidine. In other embodiments X_1 is cytosine and/or X_2 is guanine. In other embodiments, as discussed herein, the nucleic acid may encompass other motifs, provided it is long enough to do so.

In other embodiments the TG nucleic acid is represented by at least the formula:



wherein X_1 , X_2 , X_3 , and X_4 are nucleotides. In some embodiments, X_1X_2 are nucleotides selected from the group consisting of GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, TpA and TpT; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpC, ApC, GpC, TpA, ApA, and CpA; N is any nucleotide and N_1 and N_5 are nucleic acid sequences composed of any number of nucleotides

provide that the sum total of N_1 and N_5 is in the range of 9 to 19. In some embodiments, X_1X_2 are GpA or GpT and X_3X_4 are TpT. In other embodiments X_1 or X_3 or both are purines and X_2 or X_4 or both are pyrimidines or X_1X_2 are CpA and X_3 or X_4 or both are pyrimidines. In one preferred embodiment, X_3X_4 are nucleotides selected from the group consisting of: TpT, TpC and TpA.

The immunostimulatory nucleic acid may be any size (i.e., length) provided it is at least 4 nucleotides. In important embodiments, the immunostimulatory nucleic acids have a length in the range of between 6 and 100. In still other embodiments, the length is in the range of between 8 and 35 nucleotides. Preferably, the TG oligonucleotides range in size from 15 to 25 nucleotides.

The size (i.e., the number of nucleotide residues along the length of the nucleic acid) of the immunostimulatory nucleic acid may also contribute to the stimulatory activity of the nucleic acid. It has been discovered, surprisingly that even for highly

immune stimulating immunostimulatory nucleic acids, the length of the nucleic acid influences the extent of immunostimulation that can be achieved. It has been demonstrated that increasing the length of a T-rich nucleic acid up to 24 nucleotides causes increased immune stimulation. The experiments presented in the examples

demonstrate that when the length of the T-rich nucleic acid is increased from 18 to 27 nucleotides the ability of the nucleic acid to stimulate an immune response is increased significantly (compare ODN #2194, 2183, 2195, and 2196 decreasing in size from 27-18 nucleotides). Increasing the length of the nucleic acid up to 30 nucleotides had a dramatic impact on the biological properties of the nucleic acid but increasing the length beyond 30 nucleotides did not appear to further influence the immune stimulatory effect (e.g., compare ODN 2179 to 2006).

It has been shown that TG nucleic acids ranging in length from 15 to 25 nucleotides in length may exhibit an increased immune stimulation. Thus, in one aspect, the invention provides an oligonucleotide that is 15-27 nucleotides in length (i.e., an oligonucleotide that is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 nucleotides in length) that may be a T-rich nucleic acid or may be a TG nucleic acid, or may be both a T-rich and a TG nucleic acid. In one embodiment, the oligonucleotide is not a T-rich nucleic acid nor is it a TG nucleic acid. In other embodiments, the oligonucleotide does not have a CG motif. The invention similarly provides oligonucleotides that are 15-27

nucleotides in length, oligonucleotides that are 18-25 nucleotides in length, oligonucleotides that are 20-23 nucleotides in length, and oligonucleotides that are 23-25 nucleotides in length. Any of the foregoing embodiments relating to oligonucleotides 15-27 in length also relate to the oligonucleotides of these differing lengths. The invention further embraces the use of any of these foregoing oligonucleotides in the methods recited herein.

Although a maximal level of immune stimulation is achieved with some T-rich nucleic acids when the nucleic acid is 24-30 nucleotide residues in length, as well as with some TG nucleic acids that range from 15 to 25 nucleotides in length, shorter or longer immunostimulatory nucleic acids can also be used according to the methods of the invention. For facilitating uptake into cells immunostimulatory nucleic acids preferably have a minimum length of 6 nucleotide residues. Nucleic acids of any size greater than 6 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded inside of cells. Preferably the immunostimulatory nucleic acids are in the range of between 8 and 100 and in some embodiments T-rich containing immunostimulatory nucleic acids are between 24 and 40 nucleotides in length and TG containing immunostimulatory nucleic acids are between 15 and 25 nucleotides in length.

In one embodiment the T-rich nucleic acid is represented by at least the formula:



wherein X_1 , X_2 , X_3 , and X_4 are nucleotides. In one embodiment X_1X_2 is TT and/or X_3X_4 is TT. In another embodiment X_1X_2 are any one of the following nucleotides TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, GT, GG, GA, and GC, and X_3X_4 are any one of the following nucleotides TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, GT, GG, GA, and GC.

In some embodiments it is preferred that the immunostimulatory nucleic acids do not contain poly-G (CCCC), or poly-A (AAAA). In other embodiments it is preferred that the immunostimulatory nucleic acid include poly-C, poly-A, poly-G (CGCG) or multiple GAs. In particular poly-G or multiple GA motifs have dramatic effects on some immunostimulatory nucleic acids. The effect of these runs-T sequences depends in part on the status of the nucleic acid backbone. For instance, if the nucleic acid has a

phosphodiester backbone or a chimeric backbone the inclusion of these sequences in the nucleic acid will only have minimal if any effect on the biological activity of the nucleic acid. If the backbone is completely phosphorothioate (or other phosphate modification) or significantly phosphorothioate then the inclusion of these sequences may have more influence on the biological activity or the kinetics of the biological activity, causing a decrease in potency of the T-rich and TG immunostimulatory nucleic acids.

Although C-rich nucleic acids have been demonstrated to have immune stimulating properties, insertion of Poly-C sequences into a T-rich nucleic acid in a manner that would reduce the relative proportion of T nucleotides in the nucleic acid can have a negative impact on the nucleic acid. Although applicants are not bound by a proposed mechanism, it is believed that the immune system has developed a mechanism for distinguishing nucleic acids having different nucleotide properties, possibly resulting from different sets of binding proteins which recognize different sequences or specific binding proteins which recognize all the immunostimulatory sequences but with different affinities. In general nucleic acids including unmethylated CpG motifs are the most immunostimulatory, followed by T-rich nucleic acids, TG nucleic acids and C-rich nucleic acids. This generalization, however, has many exceptions. For instance a strong T-rich nucleic acid like SEQ ID NO.: 886 is more immune stimulatory in some assays than some CpG containing nucleic acids (e.g., a phosphorothioate CpG nucleic acid containing a single CpG motif).

It has also been discovered that the addition of a poly-A tail to an immunostimulatory nucleic acid can enhance the activity of the nucleic acid. It was discovered that when a highly immune stimulatory CpG nucleic acid (SEQ ID NO.: 246) was modified with the addition of a poly-A tail (AAAAAA) or a poly-T tail (TTTTTT), the resultant oligonucleotides increased in immune stimulatory activity. The ability of the poly-A tail and the poly-T tail to increase the immunostimulating properties of the oligonucleotide was very similar. SEQ ID NO.: 246 is a T-rich oligonucleotide. It is likely that if poly-A and poly-T tails are added to a nucleic acid which is not T-rich, it would have a bigger impact on the immune stimulating capability of the nucleic acid. Since the poly-T tail was added to a nucleic acid that was already highly T-rich the immune stimulating properties of the poly-T addition was diluted somewhat, although not completely. This finding has important implications for the use of poly-A regions.

Thus in some embodiments the immunostimulatory nucleic acids include a poly-A region and in other embodiments they do not.

Some of the immunostimulatory nucleic acids of the invention include one or more CG motifs. The presence of CG motifs in the immunostimulatory nucleic acids also has an influence on the biological activity of the nucleic acids. If the total length of an immunostimulatory nucleic acid is 20 nucleotide residues or less, then CpG motifs are important in determining the immune effect of the nucleic acid, and methylation of these motifs reduces the potency of the immune stimulatory effects of the nucleic acid. If the length of the immunostimulatory nucleic acid is increased to 24, then the immune stimulatory effects of the nucleic acid become less dependent on the CpG motifs, and are no longer abolished by methylation of the CpG motifs or by their inversion to GC dinucleotides, provided the other immune-stimulatory properties described herein are present.

For example, ODN 2006 (SEQ ID NO:246) is a highly immune stimulatory T-rich nucleic acid of 24 nucleotide residues in length with four CpG dinucleotides.

However, ODN 2117 (SEQ ID NO:338), in which the CpG motifs are methylated is also highly immune stimulatory. ODN 2137 (SEQ ID NO:886), in which the CpG motifs of ODN 2006 are inverted to CpC, and which as a result possesses six TG dinucleotides is also immune stimulatory. The immune stimulatory effects of nucleic acids such as ODN

2117 and 2137 are regulated by their T and TG content. Each of these three nucleic acids is T-rich and ODN 2137 is additionally TG rich. If their T content is reduced by inserting other bases such as A (ODN 2117 (SEQ ID NO:338)) or if their TG content is reduced by substituting TG with AG, then the immune stimulatory effects are somewhat reduced. In another example, a nucleic acid 24 nucleotides in length in which all of the positions are randomized has only a modest immune stimulatory effect (ODN 2182 (SEQ ID NO:432)). Likewise, a nucleic acid 24 nucleotides in length with other nucleotide compositions have variable immune stimulatory effects, depending on their T content (ODN 2188 (SEQ ID NO:903), 2189 (SEQ ID NO:906), 2190 (SEQ ID

NO:907), 2191 (SEQ ID NO:908), 2193 (SEQ ID NO:910), 2183 (SEQ ID NO:433), and 2178 (SEQ ID NO:428)). ODN 2190 which contains TGT motifs is more immune stimulatory than ODN 2202 which possesses TGG motifs. Thus, in some embodiments, TGT motifs are preferred. In still other embodiments, the number of TG motifs is

important in that an increase in the number of TG motifs leads to an increase in immune stimulation. Some preferred TG nucleic acids contain at least three TG motifs.

Examples of CpG nucleic acids include but are not limited to those listed in Table A, such as SEQ ID NO: 1, 3, 4, 14-16, 18-24, 28, 29, 33-46, 49, 50, 52-56, 58, 64-67, 69, 71, 72, 76-87, 90, 91, 93, 94, 96, 98, 102-124, 126-128, 131-133, 136-141, 146-150, 152-153, 155-171, 173-178, 180-186, 188-198, 201, 203-214, 216-220, 223, 224, 227-240, 242-256, 258, 260-263, 270-273, 275, 277-281, 286-287, 292, 295-296, 300, 302, 305-307, 309-312, 314-317, 320-327, 329, 335, 337-341, 343-352, 354, 357, 361-365, 367-369, 373-376, 378-385, 388-392, 394, 395, 399, 401-404, 406-426, 429-433, 434-437, 439, 441-443, 445, 447, 448, 450, 453-456, 460-464, 466-469, 472-475, 477, 478, 480, 483-485, 488, 489, 492, 493, 495-502, 504-505, 507-509, 511, 513-529, 532-541, 543-555, 564-566, 568-576, 578, 580, 599, 601-605, 607-611, 613-615, 617, 619-622, 625-646, 648-650, 653-664, 666-697, 699-706, 708, 709, 711-716, 718-732, 736, 737, 739-744, 746, 747, 749-761, 763, 766-767, 769, 772-779, 781-783, 785-786, 790-792, 798-799, 804-808, 810, 815, 817, 818, 820-832, 835-846, 849-850, 853-859, 862, 865, 872, 874-877, 879-881, 883-885, 888-904, and 909-913.

In some embodiments of the invention the immunostimulatory nucleic acids include CpG dinucleotides and in other embodiments the immunostimulatory nucleic acids are free of CpG dinucleotides. The CpG dinucleotides may be methylated or unmethylated. A nucleic acid containing at least one unmethylated CpG dinucleotide is

a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing an unmethylated 5' cytosine followed by 3' guanosine and linked by a phosphate bond) and activates the immune system. A nucleic acid containing at least one methylated CpG dinucleotide is a nucleic acid which contains a methylated cytosine-guanine dinucleotide sequence (i.e., a methylated 5' cytosine followed by a 3' guanosine and linked by a phosphate bond).

Examples of T rich nucleic acids that are free of CpG nucleic acids include but are not limited to those listed in Table A, such as SEQ ID NO: 59-63, 73-75, 142, 215, 226, 241, 267-269, 282, 301, 304, 330, 342, 358, 370-372, 393, 433, 471, 479, 486, 491, 497, 503, 556-558, 567, 694, 793-794, 797, 833, 852, 861, 867, 868, 882, 886, 905, 907, 908, and 910-913. Examples of T rich nucleic acids that include CpG nucleic acids include but are not limited to those listed in Table A, such as SEQ ID NO: 64, 98, 112,

- 33 -

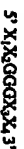
146, 183, 204, 208, 214, 224, 233, 244, 246, 247, 258, 262, 263, 265, 270-273, 300, 305, 316, 317, 343, 344, 350, 352, 354, 374, 376, 392, 407, 411-413, 429-432, 434, 435, 443, 474, 475, 498-501, 518, 587, 692, 693, 804, 862, 883, 884, 888, 890, and 891.

The immunostimulatory nucleic acids can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased immuno activity. Thus in some aspects of the invention it is preferred that the nucleic acid be single stranded and in other aspects it is preferred that the nucleic acid be double stranded.

The term T-rich nucleic acid and TG nucleic acid, as used herein, refer to an immunostimulatory T-rich nucleic acid and an immunostimulatory TG nucleic acid, respectively, unless otherwise indicated. The T-rich nucleic acid sequences of the invention are those broadly described above as well as the nucleic acids shown in Table A that have at least one poly T motif and/or have a composition of greater than 25% T or preferably 35% nucleotide residues. The C-rich nucleic acids are those having at least one and preferably at least two poly-C regions. The TG nucleic acids of the invention are those broadly described above as well as the specific nucleic acids shown in Table A that have at least one TG motif.

The nucleic acids of the invention may, but need not, also include a poly G motif. Poly G containing nucleic acids are also immunostimulatory. A variety of references, including Plascinsky and Reich, 1993 *Mol. Biol. Reports*, 18:217-221; Krieger and Herz, 1994, *Ann. Rev. Biochem.*, 63:601-637; Maseya et al., 1993, *PNAS*, 90:3745-3749; Wyatt et al., 1994, *PNAS*, 91:1356-1360; Rando and Hogan, 1998, in *Applied Antisense Oligonucleotide Technology*, ed. Krieger and Stein, p. 335-352; and Kimura et al., 1994, *J. Biochem.*, 116, 991-994 also describe the immunostimulatory properties of poly G nucleic acids.

Poly G nucleic acids preferably are nucleic acids having the following formula:



wherein X_1 , X_2 , X_3 , and X_4 are nucleotides. In preferred embodiments at least one of X_3 and X_4 are a G. In other embodiments both of X_3 and X_4 are a G. In yet other embodiments the preferred formula is 5' GGGNGGG 3', or 5' GGGNGGGNGGG 3' wherein N represents between 0 and 20 nucleotides. In other embodiments the poly G nucleic acid is free of unmethylated CG dinucleotides, such as, for example, the nucleic

- 34 -

acids listed below as SEQ ID NO: 5, 6, 73, 215, 267-269, 276, 282, 288, 297-299, 355, 359, 386, 397, 444, 476, 531, 557-559, 733, 768, 795, 796, 914-925, 928-931, 933-936, and 938. In other embodiments the poly G nucleic acid includes at least one unmethylated CG dinucleotide, such as, for example, the nucleic acids listed above as SEQ ID NO: 67, 80-82, 141, 147, 148, 173, 178, 183, 185, 214, 224, 264, 265, 315, 329, 434, 435, 475, 519, 521-524, 526, 527, 535, 554, 565, 609, 628, 660, 661, 662, 725, 767, 825, 856, 857, 876, 892, 909, 926, 927, 932, and 937.

The terms "nucleic acid" and "oligonucleotide" are used interchangeably to mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymidine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the terms refer to oligonucleotides as well as oligodeoxyribonucleotides. The terms shall also include polynucleotides (i.e. a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by nucleic acid synthesis).

The terms nucleic acid and oligonucleotide also encompass nucleic acids or oligonucleotides with substitutions or modifications, such as in the bases and/or sugars. For example, they include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose instead of ribose. Thus the nucleic acids may be heterogeneous in backbone composition thereby combining any possible combination of polymer units linked together such as peptide- nucleic acids (which have amino acid backbone with nucleic acid bases). In some embodiments, the nucleic acids are homogeneous in backbone composition. Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne modified bases (Wagner et al., *Nature Biotechnology* 14:840-844, 1996). Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymidine, 5-methylcytosine, 2-aminoguanine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and

non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties. Other such modifications are well known to those of skill in the art.

For use in the instant invention, the nucleic acids of the invention can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Besauage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4031-4054, 1986; Froehner *et al.*, *Nucl. Acid. Res.* 14:5399-5407, 1986; Garegg *et al.*, *Tet. Let.* 27:4055-4058, 1986; Garfney *et al.*, *Tet. Let.* 22:2619-2622, 1988).

These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These nucleic acids are referred to as synthetic nucleic acids.

Alternatively, T-rich and/or TG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1989) and separated into smaller pieces or administered whole. Nucleic acids can be prepared from existing nucleic acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases. Nucleic acids prepared in this manner are referred to as isolated nucleic acid. An isolated nucleic acid generally refers to a nucleic acid which is separated from components which it is normally associated with in nature. As an example, an isolated nucleic acid may be one which is separated from a cell, from a nucleus, from mitochondria or from chromatin. The terms Py-rich nucleic acids and TG nucleic acids encompasses both synthetic and isolated Py-rich nucleic acids and TG nucleic acids.

For use *in vivo*, the Py-rich and TG nucleic acids may optionally be relatively resistant to degradation (e.g., are stabilized). A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to *in vivo* degradation (e.g. via an *exo- or endo-nuclease*). Stabilization can be a function of length or secondary structure. Nucleic acids that are (are to hundreds of kbs long are relatively resistant to *in vivo* degradation. For shorter nucleic acids, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic acid has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the nucleic acid becomes stabilized and therefore exhibits more activity.

Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. Preferred stabilized nucleic acids of the instant invention have a modified backbone. It has been demonstrated that modification of the nucleic acid backbone provides enhanced activity of the Py-rich and TG nucleic acids when administered *in vivo*. These stabilized structures are preferred because the Py-rich and TG molecules of the invention have at least a partial modified backbone. Py-rich and TG constructs having phosphorothioate linkages provide maximal activity and protect the nucleic acid from degradation by intracellular *exo- and endo-nucleases*. Other modified nucleic acids include phosphodiester modified nucleic acids, combinations of phosphodiester and phosphorothioate nucleic acid, methylphosphonate, methylphosphorothioate, phosphorodithioate, *p-ethoxy*, and combinations thereof. Each of these combinations and their particular effects on immune cells is discussed in more detail with respect to CpG nucleic acids in PCT Published Patent Applications PCT/US95/01570 (WO 96/02555) and PCT/US97/19791 (WO 98/18810) claiming priority to U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and October 30, 1997 respectively, the entire contents of which are hereby incorporated by reference. It is believed that these modified nucleic acids may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

The compositions of the invention may optionally be chimeric oligonucleotides. The chimeric oligonucleotides are oligonucleotides having a formula: 5' Y₁NZ_NY₂ 3', Y₁ and Y₂ are nucleic acid molecules having between 1 and 10 nucleotides. Y₁ and Y₂ each include at least one modified internucleotide linkage. Since at least 2 nucleotides of the chimeric oligonucleotides include backbone modifications these nucleic acids are an example of one type of "stabilized immunostimulatory nucleic acids."

With respect to the chimeric oligonucleotides, Y₁ and Y₂ are considered independent of one another. This means that each of Y₁ and Y₂ may or may not have different sequences and different backbone linkages from one another in the same molecule. The sequences vary, but in some cases Y₁ and Y₂ have a poly-G sequence. A poly-G sequence refers to at least 3 Gs in a row. In other embodiments the poly-G sequence refers to at least 4, 5, 6, 7, or 8 Gs in a row. In other embodiments Y₁ and Y₂ may be TCGTCG, TCGTCGT, or TCGTCGTT (SEQ ID NO:1145). Y₁ and Y₂ may also

have a poly-C, poly-T, or poly-A sequence. In some embodiments Y₁ and/or Y₂ have between 3 and 8 nucleotides.

N₁ and N₂ are nucleic acid molecules having between 0 and 5 nucleotides as long as N₁ZN₂ has at least 6 nucleotides in total. The nucleotides of N₁ZN₂ have a phosphodiester backbone and do not include nucleic acids having a modified backbone.

Z is an immunostimulatory nucleic acid motif but does not include a CG. For instance, Z may be a nucleic acid a T-rich sequence, e.g. including a TTTT motif or a sequence wherein at least 50% of the bases of the sequence are Ts or Z may be a TG sequence.

The center nucleotides (N₁ZN₂) of the formula Y₁N₁ZN₂Y₂ have phosphodiester internucleotide linkages and Y₁ and Y₂ have at least one, but may have more than one or even may have all modified internucleotide linkages. In preferred embodiments Y₁ and/or Y₂ have at least two or between two and five modified internucleotide linkages or Y₁ has two modified internucleotide linkages and Y₂ has five modified internucleotide linkages or Y₁ has five modified internucleotide linkages and Y₂ has two modified internucleotide linkages. The modified internucleotide linkages, in some embodiments is a phosphorothioate modified linkage, a phosphorodithioate modified linkage or a p-ethoxy modified linkage.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidates or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphonates (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, B. and Peyman, A., *Chem. Rev.* 20:544, 1990; Goodchild, J., *Bioconjugates Chem.* 1:165, 1990).

Other stabilized nucleic acids include: ionomic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphonates, in which the charged oxygen moiety is alkylated. Nucleic acids which contain diol, such as tetrahydropyranol or

hexaethyleneol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

In the case when the Py-rich or TG nucleic acid is administered in conjunction with an antigen which is encoded in a nucleic acid vector, it is preferred that the backbone of the Py-rich or TG nucleic acid be a chimeric combination of phosphodiester and phosphorothioate (or other phosphate modification). The cell may have a problem taking up a plasmid vector in the presence of completely phosphorothioate nucleic acid. Thus when both a vector and a nucleic acid are delivered to a subject, it is preferred that the nucleic acid have a chimeric backbone or have a phosphorothioate backbone but that the plasmid be associated with a vehicle that delivers it directly into the cell, thus avoiding the need for cellular uptake. Such vehicles are known in the art and include, for example, liposomes and gene guns.

The nucleic acids described herein as well as various control nucleic acids are presented below in Table A.

15

Table A

SEQ ID NO:	DNV SEQUENCE	BACKBONE
1	tcctccagctggcccat	a
2	ataatccagctggaccag	a
3	ataatcgagcttcaagcag	a
4	taccgctggaccctct	a
5	ggggaaggt	a
6	ggggaaggt	a
7	ggggaaggt	a
8	tcacatggttccctgact	a
9	tcacatggttccctgact	a
10	tcacatggttccctgact	a
11	tcacatggttccctgact	a
12	gcctagagcttccctgact	a
13	gcctagagcttccctgact	a
14	gcctagagcttccctgact	a
15	gcctagagcttccctgact	a
16	gcctagagcttccctgact	a
17	gcctagagcttccctgact	a
18	gcctagagcttccctgact	a
19	gcctagagcttccctgact	a
20	gcctagagcttccctgact	a
21	gcctagagcttccctgact	a
22	gcctagagcttccctgact	a
23	gcctagagcttccctgact	a
24	gcctagagcttccctgact	a
25	gcctagagcttccctgact	a
26	gcctagagcttccctgact	a

27	tcctgaggttcctgagtt	0
28	gaggaagtgagagagtt	0
29	gttaattctctcctgagtt	0
30	ataata	0
31	accacac	0
32	ctctca	0
33	tcctgag	0
34	tcctgag	0
35	tcctgag	0
36	tcctgaggttcctgagtt	008
37	tcctgaggttcctgagtt	008
38	tcctgaggttcctgagtt	008
39	tcctgaggttcctgagtt	008
40	tcctgaggttcctgagtt	008
41	tcctgaggttcctgagtt	008
42	tcctgaggttcctgagtt	008
43	tcctgaggttcctgagtt	008
44	tcctgaggttcctgagtt	008
45	tcctgaggttcctgagtt	008
46	tcctgaggttcctgagtt	008
47	tcctgaggttcctgagtt	008
48	tcctgaggttcctgagtt	008
49	tcctgaggttcctgagtt	008
50	tcctgaggttcctgagtt	008
51	tcctgaggttcctgagtt	008
52	tcctgaggttcctgagtt	008
53	tcctgaggttcctgagtt	008
54	tcctgaggttcctgagtt	008
55	tcctgaggttcctgagtt	008
56	tcctgaggttcctgagtt	008
57	tcctgaggttcctgagtt	008
58	tcctgaggttcctgagtt	008
59	tcctgaggttcctgagtt	008
60	tcctgaggttcctgagtt	008
61	tcctgaggttcctgagtt	008
62	tcctgaggttcctgagtt	008
63	tcctgaggttcctgagtt	008
64	tcctgaggttcctgagtt	008
65	tcctgaggttcctgagtt	008
66	tcctgaggttcctgagtt	008
67	tcctgaggttcctgagtt	008
68	tcctgaggttcctgagtt	008
69	tcctgaggttcctgagtt	008
70	tcctgaggttcctgagtt	008
71	tcctgaggttcctgagtt	008
72	tcctgaggttcctgagtt	008
73	tcctgaggttcctgagtt	008
74	tcctgaggttcctgagtt	008
75	tcctgaggttcctgagtt	008
76	tcctgaggttcctgagtt	008
77	tcctgaggttcctgagtt	008

78	tcctgaggttcctgagtt	0
79	tcctgaggttcctgagtt	0
80	tcctgaggttcctgagtt	008
81	tcctgaggttcctgagtt	008
82	tcctgaggttcctgagtt	008
83	tcctgaggttcctgagtt	008
84	tcctgaggttcctgagtt	008
85	tcctgaggttcctgagtt	008
86	tcctgaggttcctgagtt	008
87	tcctgaggttcctgagtt	008
88	tcctgaggttcctgagtt	008
89	tcctgaggttcctgagtt	008
90	tcctgaggttcctgagtt	008
91	tcctgaggttcctgagtt	008
92	tcctgaggttcctgagtt	008
93	tcctgaggttcctgagtt	008
94	tcctgaggttcctgagtt	008
95	tcctgaggttcctgagtt	008
96	tcctgaggttcctgagtt	008
97	tcctgaggttcctgagtt	008
98	tcctgaggttcctgagtt	008
99	tcctgaggttcctgagtt	008
100	tcctgaggttcctgagtt	008
101	tcctgaggttcctgagtt	008
102	tcctgaggttcctgagtt	008
103	tcctgaggttcctgagtt	008
104	tcctgaggttcctgagtt	008
105	tcctgaggttcctgagtt	008
106	tcctgaggttcctgagtt	008
107	tcctgaggttcctgagtt	008
108	tcctgaggttcctgagtt	008
109	tcctgaggttcctgagtt	008
110	tcctgaggttcctgagtt	008
111	tcctgaggttcctgagtt	008
112	tcctgaggttcctgagtt	008
113	tcctgaggttcctgagtt	008
114	tcctgaggttcctgagtt	008
115	tcctgaggttcctgagtt	008
116	tcctgaggttcctgagtt	008
117	tcctgaggttcctgagtt	008
118	tcctgaggttcctgagtt	008
119	tcctgaggttcctgagtt	008
120	tcctgaggttcctgagtt	008
121	tcctgaggttcctgagtt	008
122	tcctgaggttcctgagtt	008
123	tcctgaggttcctgagtt	008
124	tcctgaggttcctgagtt	008
125	tcctgaggttcctgagtt	008
126	tcctgaggttcctgagtt	008
127	tcctgaggttcctgagtt	008
128	tcctgaggttcctgagtt	008

129	ctcaggttctccacaaag	0
130	ctcaggttctccacaaag	0
131	ctcaggttctccacaaag	0
132	ctcaggttctccacaaag	0
133	ctcaggttctccacaaag	0
134	ctcaggttctccacaaag	0
135	ctcaggttctccacaaag	0
136	ctcaggttctccacaaag	0
137	ctcaggttctccacaaag	0
138	ctcaggttctccacaaag	0
139	ctcaggttctccacaaag	0
140	ctcaggttctccacaaag	0
141	ctcaggttctccacaaag	0
142	ctcaggttctccacaaag	0
143	ctcaggttctccacaaag	0
144	ctcaggttctccacaaag	0
145	ctcaggttctccacaaag	0
146	ctcaggttctccacaaag	0
147	ctcaggttctccacaaag	0
148	ctcaggttctccacaaag	0
149	ctcaggttctccacaaag	0
150	ctcaggttctccacaaag	0
151	ctcaggttctccacaaag	0
152	ctcaggttctccacaaag	0
153	ctcaggttctccacaaag	0
154	ctcaggttctccacaaag	0
155	ctcaggttctccacaaag	0
156	ctcaggttctccacaaag	0
157	ctcaggttctccacaaag	0
158	ctcaggttctccacaaag	0
159	ctcaggttctccacaaag	0
160	ctcaggttctccacaaag	0
161	ctcaggttctccacaaag	0
162	ctcaggttctccacaaag	0
163	ctcaggttctccacaaag	0
164	ctcaggttctccacaaag	0
165	ctcaggttctccacaaag	0
166	ctcaggttctccacaaag	0
167	ctcaggttctccacaaag	0
168	ctcaggttctccacaaag	0
169	ctcaggttctccacaaag	0
170	ctcaggttctccacaaag	0
171	ctcaggttctccacaaag	0
172	ctcaggttctccacaaag	0
173	ctcaggttctccacaaag	0
174	ctcaggttctccacaaag	0
175	ctcaggttctccacaaag	0
176	ctcaggttctccacaaag	0
177	ctcaggttctccacaaag	0
178	ctcaggttctccacaaag	0
179	ctcaggttctccacaaag	0

180	ctcaggttctccacaaag	0
181	ctcaggttctccacaaag	0
182	ctcaggttctccacaaag	0
183	ctcaggttctccacaaag	0
184	ctcaggttctccacaaag	0
185	ctcaggttctccacaaag	0
186	ctcaggttctccacaaag	0
187	ctcaggttctccacaaag	0
188	ctcaggttctccacaaag	0
189	ctcaggttctccacaaag	0
190	ctcaggttctccacaaag	0
191	ctcaggttctccacaaag	0
192	ctcaggttctccacaaag	0
193	ctcaggttctccacaaag	0
194	ctcaggttctccacaaag	0
195	ctcaggttctccacaaag	0
196	ctcaggttctccacaaag	0
197	ctcaggttctccacaaag	0
198	ctcaggttctccacaaag	0
199	ctcaggttctccacaaag	0
200	ctcaggttctccacaaag	0
201	ctcaggttctccacaaag	0
202	ctcaggttctccacaaag	0
203	ctcaggttctccacaaag	0
204	ctcaggttctccacaaag	0
205	ctcaggttctccacaaag	0
206	ctcaggttctccacaaag	0
207	ctcaggttctccacaaag	0
208	ctcaggttctccacaaag	0
209	ctcaggttctccacaaag	0
210	ctcaggttctccacaaag	0
211	ctcaggttctccacaaag	0
212	ctcaggttctccacaaag	0
213	ctcaggttctccacaaag	0
214	ctcaggttctccacaaag	0
215	ctcaggttctccacaaag	0
216	ctcaggttctccacaaag	0
217	ctcaggttctccacaaag	0
218	ctcaggttctccacaaag	0
219	ctcaggttctccacaaag	0
220	ctcaggttctccacaaag	0
221	ctcaggttctccacaaag	0
222	ctcaggttctccacaaag	0
223	ctcaggttctccacaaag	0
224	ctcaggttctccacaaag	0
225	ctcaggttctccacaaag	0
226	ctcaggttctccacaaag	0
227	ctcaggttctccacaaag	0
228	ctcaggttctccacaaag	0
229	ctcaggttctccacaaag	0
230	ctcaggttctccacaaag	0

231	tcaatgagcttactgctgtt	a
232	tcaatgagcttactgctgtt	a
233	tcaatgagcttactgctgtt	a
234	tcaatgagcttactgctgtt	a
235	tcaatgagcttactgctgtt	a
236	tcaatgagcttactgctgtt	a
237	tcaatgagcttactgctgtt	a
238	tcaatgagcttactgctgtt	a
239	tcaatgagcttactgctgtt	a
240	tcaatgagcttactgctgtt	a
241	tcaatgagcttactgctgtt	a
242	tcaatgagcttactgctgtt	a
243	tcaatgagcttactgctgtt	a
244	tcaatgagcttactgctgtt	a
245	tcaatgagcttactgctgtt	a
246	tcaatgagcttactgctgtt	a
247	tcaatgagcttactgctgtt	a
248	tcaatgagcttactgctgtt	a
249	tcaatgagcttactgctgtt	a
250	tcaatgagcttactgctgtt	a
251	tcaatgagcttactgctgtt	a
252	tcaatgagcttactgctgtt	a
253	tcaatgagcttactgctgtt	a
254	tcaatgagcttactgctgtt	a
255	tcaatgagcttactgctgtt	a
256	tcaatgagcttactgctgtt	a
257	tcaatgagcttactgctgtt	a
258	tcaatgagcttactgctgtt	a
259	tcaatgagcttactgctgtt	a
260	tcaatgagcttactgctgtt	a
261	tcaatgagcttactgctgtt	a
262	tcaatgagcttactgctgtt	a
263	tcaatgagcttactgctgtt	a
264	tcaatgagcttactgctgtt	a
265	tcaatgagcttactgctgtt	a
266	tcaatgagcttactgctgtt	a
267	tcaatgagcttactgctgtt	a
268	tcaatgagcttactgctgtt	a
269	tcaatgagcttactgctgtt	a
270	tcaatgagcttactgctgtt	a
271	tcaatgagcttactgctgtt	a
272	tcaatgagcttactgctgtt	a
273	tcaatgagcttactgctgtt	a
274	tcaatgagcttactgctgtt	a
275	tcaatgagcttactgctgtt	a
276	tcaatgagcttactgctgtt	a
277	tcaatgagcttactgctgtt	a
278	tcaatgagcttactgctgtt	a
279	tcaatgagcttactgctgtt	a
280	tcaatgagcttactgctgtt	a
281	tcaatgagcttactgctgtt	a

282	tcaatgagcttactgctgtt	a
283	tcaatgagcttactgctgtt	a
284	tcaatgagcttactgctgtt	a
285	tcaatgagcttactgctgtt	a
286	tcaatgagcttactgctgtt	a
287	tcaatgagcttactgctgtt	a
288	tcaatgagcttactgctgtt	a
289	tcaatgagcttactgctgtt	a
290	tcaatgagcttactgctgtt	a
291	tcaatgagcttactgctgtt	a
292	tcaatgagcttactgctgtt	a
293	tcaatgagcttactgctgtt	a
294	tcaatgagcttactgctgtt	a
295	tcaatgagcttactgctgtt	a
296	tcaatgagcttactgctgtt	a
297	tcaatgagcttactgctgtt	a
298	tcaatgagcttactgctgtt	a
299	tcaatgagcttactgctgtt	a
300	tcaatgagcttactgctgtt	a
301	tcaatgagcttactgctgtt	a
302	tcaatgagcttactgctgtt	a
303	tcaatgagcttactgctgtt	a
304	tcaatgagcttactgctgtt	a
305	tcaatgagcttactgctgtt	a
306	tcaatgagcttactgctgtt	a
307	tcaatgagcttactgctgtt	a
308	tcaatgagcttactgctgtt	a
309	tcaatgagcttactgctgtt	a
310	tcaatgagcttactgctgtt	a
311	tcaatgagcttactgctgtt	a
312	tcaatgagcttactgctgtt	a
313	tcaatgagcttactgctgtt	a
314	tcaatgagcttactgctgtt	a
315	tcaatgagcttactgctgtt	a
316	tcaatgagcttactgctgtt	a
317	tcaatgagcttactgctgtt	a
318	tcaatgagcttactgctgtt	a
319	tcaatgagcttactgctgtt	a
320	tcaatgagcttactgctgtt	a
321	tcaatgagcttactgctgtt	a
322	tcaatgagcttactgctgtt	a
323	tcaatgagcttactgctgtt	a
324	tcaatgagcttactgctgtt	a
325	tcaatgagcttactgctgtt	a
326	tcaatgagcttactgctgtt	a
327	tcaatgagcttactgctgtt	a
328	tcaatgagcttactgctgtt	a
329	tcaatgagcttactgctgtt	a
330	tcaatgagcttactgctgtt	a
331	tcaatgagcttactgctgtt	a
332	tcaatgagcttactgctgtt	a

[illegible]

384	gtctgcacagatctctgaa	
385	gtctgcacagcttactctga	
386	gggggtccctctctctctctgg	
387	gggggtccctggagctctgc	
388	ggagagcgtggatctccat	
389	tccatgttcgtctggatggt	
390	ctctctggatctctggagatga	
391	aggtctgcacggatctctga	
392	accatggatctctgttcccttc	
393	accatggatctctctctccct	
394	atggatctggatctctctctga	
395	ctctctcagctctctctctga	
396	ctctctcagctctctctctga	
397	tcccttgatctctctccacat	
398	ggatccacatctctctctg	
399	gtctcaggtctctctggatga	
400	ctctctctctctctctctctg	
401	ggggggatctctctctctctg	
402	gggtctccatctctctctctg	
403	ggatctctggatctctctctctg	
404	ccatgtgtgtatctctctctctg	
405	ccaggtgtctctctctctctg	
406	tctccatctctctctctctg	
407	tctccatctctctctctctg	
408	tctccatctctctctctctg	
409	tctccatctctctctctctg	
410	atctctctctctctctctg	
411	tctctctctctctctctctg	
412	tctctctctctctctctctg	
413	tctctctctctctctctctg	
414	tctctctctctctctctctg	
415	tctctctctctctctctctg	
416	tctctctctctctctctctg	
417	atctctctctctctctctg	
418	atctctctctctctctctg	
419	tctctctctctctctctctg	
420	tctctctctctctctctctg	
421	tctctctctctctctctctg	
422	tctctctctctctctctctg	
423	tctctctctctctctctctg	
424	tctctctctctctctctctg	
425	tctctctctctctctctctg	
426	tctctctctctctctctctg	
427	atctctctctctctctctg	
428	ccctctctctctctctctg	
429	tctctctctctctctctctg	
430	tctctctctctctctctctg	
431	tctctctctctctctctctg	
432	tctctctctctctctctctg	
433	tctctctctctctctctctg	
434	gggtctctctctctctctg	

537	gategctgataagctg	808
538	gtcgtccctgctgcttc	808
539	tctgtccctgctgcttc	808
540	ctggtccctgctgcttc	808
541	gtcgtccctgctgcttc	808
542	ctggtccctgctgcttc	808
543	ctggtccctgctgcttc	808
544	ctggtccctgctgcttc	808
545	ctggtccctgctgcttc	808
546	ctggtccctgctgcttc	808
547	ctggtccctgctgcttc	808
548	ctggtccctgctgcttc	808
549	ctggtccctgctgcttc	808
550	ctggtccctgctgcttc	808
551	ctggtccctgctgcttc	808
552	ctggtccctgctgcttc	808
553	ctggtccctgctgcttc	808
554	ctggtccctgctgcttc	808
555	ctggtccctgctgcttc	808
556	ctggtccctgctgcttc	808
557	ctggtccctgctgcttc	808
558	ctggtccctgctgcttc	808
559	ctggtccctgctgcttc	808
560	ctggtccctgctgcttc	808
561	ctggtccctgctgcttc	808
562	ctggtccctgctgcttc	808
563	ctggtccctgctgcttc	808
564	ctggtccctgctgcttc	808
565	ctggtccctgctgcttc	808
566	ctggtccctgctgcttc	808
567	ctggtccctgctgcttc	808
568	ctggtccctgctgcttc	808
569	ctggtccctgctgcttc	808
570	ctggtccctgctgcttc	808
571	ctggtccctgctgcttc	808
572	ctggtccctgctgcttc	808
573	ctggtccctgctgcttc	808
574	ctggtccctgctgcttc	808
575	ctggtccctgctgcttc	808
576	ctggtccctgctgcttc	808
577	ctggtccctgctgcttc	808
578	ctggtccctgctgcttc	808
579	ctggtccctgctgcttc	808
580	ctggtccctgctgcttc	808
581	ctggtccctgctgcttc	808
582	ctggtccctgctgcttc	808
583	ctggtccctgctgcttc	808
584	ctggtccctgctgcttc	808
585	ctggtccctgctgcttc	808
586	ctggtccctgctgcttc	808
587	ctggtccctgctgcttc	808

588	ctggtccctgctgcttc	808
589	ctggtccctgctgcttc	808
590	ctggtccctgctgcttc	808
591	ctggtccctgctgcttc	808
592	ctggtccctgctgcttc	808
593	ctggtccctgctgcttc	808
594	ctggtccctgctgcttc	808
595	ctggtccctgctgcttc	808
596	ctggtccctgctgcttc	808
597	ctggtccctgctgcttc	808
598	ctggtccctgctgcttc	808
599	ctggtccctgctgcttc	808
600	ctggtccctgctgcttc	808
601	ctggtccctgctgcttc	808
602	ctggtccctgctgcttc	808
603	ctggtccctgctgcttc	808
604	ctggtccctgctgcttc	808
605	ctggtccctgctgcttc	808
606	ctggtccctgctgcttc	808
607	ctggtccctgctgcttc	808
608	ctggtccctgctgcttc	808
609	ctggtccctgctgcttc	808
610	ctggtccctgctgcttc	808
611	ctggtccctgctgcttc	808
612	ctggtccctgctgcttc	808
613	ctggtccctgctgcttc	808
614	ctggtccctgctgcttc	808
615	ctggtccctgctgcttc	808
616	ctggtccctgctgcttc	808
617	ctggtccctgctgcttc	808
618	ctggtccctgctgcttc	808
619	ctggtccctgctgcttc	808
620	ctggtccctgctgcttc	808
621	ctggtccctgctgcttc	808
622	ctggtccctgctgcttc	808
623	ctggtccctgctgcttc	808
624	ctggtccctgctgcttc	808
625	ctggtccctgctgcttc	808
626	ctggtccctgctgcttc	808
627	ctggtccctgctgcttc	808
628	ctggtccctgctgcttc	808
629	ctggtccctgctgcttc	808
630	ctggtccctgctgcttc	808
631	ctggtccctgctgcttc	808
632	ctggtccctgctgcttc	808
633	ctggtccctgctgcttc	808
634	ctggtccctgctgcttc	808
635	ctggtccctgctgcttc	808
636	ctggtccctgctgcttc	808
637	ctggtccctgctgcttc	808
638	ctggtccctgctgcttc	808

639	taacggt		0
640	taacggt		0
641	taacggt		0
642	gacgga		0
643	gacgga		0
644	gacgga		0
645	gacgga		0
646	gacgga		0
647	gacgga		0
648	gacgga		0
649	gacgga		0
650	gacgga		0
651	gacgga		0
652	gacgga		0
653	gacgga		0
654	gacgga		0
655	gacgga		0
656	gacgga		0
657	gacgga		0
658	gacgga		0
659	gacgga		0
660	gacgga		0
661	gacgga		0
662	gacgga		0
663	gacgga		0
664	gacgga		0
665	gacgga		0
666	gacgga		0
667	gacgga		0
668	gacgga		0
669	gacgga		0
670	gacgga		0
671	gacgga		0
672	gacgga		0
673	gacgga		0
674	gacgga		0
675	gacgga		0
676	gacgga		0
677	gacgga		0
678	gacgga		0
679	gacgga		0
680	gacgga		0
681	gacgga		0
682	gacgga		0
683	gacgga		0
684	gacgga		0
685	gacgga		0
686	gacgga		0
687	gacgga		0
688	gacgga		0
689	gacgga		0

690	gacgga		0
691	gacgga		0
692	gacgga		0
693	gacgga		0
694	gacgga		0
695	gacgga		0
696	gacgga		0
697	gacgga		0
698	gacgga		0
699	gacgga		0
700	gacgga		0
701	gacgga		0
702	gacgga		0
703	gacgga		0
704	gacgga		0
705	gacgga		0
706	gacgga		0
707	gacgga		0
708	gacgga		0
709	gacgga		0
710	gacgga		0
711	gacgga		0
712	gacgga		0
713	gacgga		0
714	gacgga		0
715	gacgga		0
716	gacgga		0
717	gacgga		0
718	gacgga		0
719	gacgga		0
720	gacgga		0
721	gacgga		0
722	gacgga		0
723	gacgga		0
724	gacgga		0
725	gacgga		0
726	gacgga		0
727	gacgga		0
728	gacgga		0
729	gacgga		0
730	gacgga		0
731	gacgga		0
732	gacgga		0
733	gacgga		0
734	gacgga		0
735	gacgga		0
736	gacgga		0
737	gacgga		0
738	gacgga		0
739	gacgga		0
740	gacgga		0

741	atgactactcaggttcgtcttctc	0
742	aagctctgactctgctt	0
743	ctctaatgctgacttctaat	0
744	atctgactatcgttgcctctc	0
745	gggtgactgtgacttctaat	0
746	tctacgtctaatctctgac	0
747	ttgagatagctctaatgacttct	0
748	ttgagatagctgacttctaat	0
749	cgtctaatgactgactgactt	0
750	gctt-gtcttctgactt	0
751	atggagagctcactgctctctc	0
752	gcttctgacttctaatgct	0
753	gcttctgacttctaatgct	0
754	tctcttctgacttctgactgct	0
755	tctcttctgacttctgactgct	0
756	gcttctgacttctgactgct	0
757	gcttctgacttctgactgct	0
758	tctcttctgacttctgactgct	0
759	tctcttctgacttctgactgct	0
760	gcttctgacttctgactgct	0
761	gcttctgacttctgactgct	0
762	tctcttctgacttctgactgct	0
763	tctcttctgacttctgactgct	0
764	atctgacttctgacttctgctc	0
765	atctgacttctgacttctgctc	0
766	gcttctgacttctgactgct	0
767	gcttctgacttctgactgct	0
768	gcttctgacttctgactgct	0
769	gcttctgacttctgactgct	0
770	gcttctgacttctgactgct	0
771	gcttctgacttctgactgct	0
772	tctcttctgacttctgactgct	0
773	gcttctgacttctgactgct	0
774	tctcttctgacttctgactgct	0
775	tctcttctgacttctgactgct	0
776	aaatctgacttctgactgct	0
777	tctcttctgacttctgactgct	0
778	gcttctgacttctgactgct	0
779	gcttctgacttctgactgct	0
780	gcttctgacttctgactgct	0
781	gcttctgacttctgactgct	0
782	gcttctgacttctgactgct	0
783	gcttctgacttctgactgct	0
784	tctcttctgacttctgactgct	0
785	tctcttctgacttctgactgct	0
786	gcttctgacttctgactgct	0
787	gcttctgacttctgactgct	0
788	gcttctgacttctgactgct	0
789	gcttctgacttctgactgct	0
790	tctcttctgacttctgactgct	0
791	tctcttctgacttctgactgct	0

792	gaagatgagctcctgaagct	0	0
793	gtttttataataatttggg	0	0
794	ttttttgtttgttggtttgct	0	0
795	ctgggggggggct	0	0
796	gggtctggggggt	0	0
797	gttttgtgttggttttgg	0	0
798	ttgtatgagctcagacttgat	0	0
799	ttatagtttgaagcttaagct	0	0
800	ttatgagagatgagacttcct	0	0
801	ttatgagagatgagacttcct	0	0
802	ttcaatttttggx	0	0
803	ggaattttttttx	0	0
804	tttgttttttttttttttttt	0	0
805	ctgggttttttttttttttt	0	0
806	ttcaatttttttttttttt	0	0
807	tttgttttttttttttttt	0	0
808	tttgttttttttttttttt	0	0
809	tttgttttttttttttttt	0	0
810	tttgttttttttttttttt	0	0
811	tttgttttttttttttttt	0	0
812	tttgttttttttttttttt	0	0
813	tttgttttttttttttttt	0	0
814	tttgttttttttttttttt	0	0
815	tttgttttttttttttttt	0	0
816	tttgttttttttttttttt	0	0
817	tttgttttttttttttttt	0	0
818	tttgttttttttttttttt	0	0
819	tttgttttttttttttttt	0	0
820	tttgttttttttttttttt	0	0
821	tttgttttttttttttttt	0	0
822	tttgttttttttttttttt	0	0
823	tttgttttttttttttttt	0	0
824	tttgttttttttttttttt	0	0
825	tttgttttttttttttttt	0	0
826	tttgttttttttttttttt	0	0
827	tttgttttttttttttttt	0	0
828	tttgttttttttttttttt	0	0
829	tttgttttttttttttttt	0	0
830	tttgttttttttttttttt	0	0
831	tttgttttttttttttttt	0	0
832	tttgttttttttttttttt	0	0
833	tttgttttttttttttttt	0	0
834	tttgttttttttttttttt	0	0
835	tttgttttttttttttttt	0	0
836	tttgttttttttttttttt	0	0
837	tttgttttttttttttttt	0	0
838	tttgttttttttttttttt	0	0
839	tttgttttttttttttttt	0	0
840	tttgttttttttttttttt	0	0
841	tttgttttttttttttttt	0	0
842	tttgttttttttttttttt	0	0

843	cszsgygcstzgc	
844	tszgcagcggctgcggtgcctgcga	o
845	tszgcagcagccgcggtgcggtgcga	o
846	csztcctgcagcttccca	o
847	tsztcctgcagcgagct	o
848	tsztcctctgcagcgagct	o
849	atszgcagcagctgcgagcgcttgc	o
850	gszgcagcagctgcggtgcctgcctat	o
851	tszgcagcagcggtgcctgcctgcctgcg	o
852	cszgcagcagcagcttgcctgcctgcgga	o
853	tszgcggtgcggtgcggtgc	ss
854	tszgcggtgcctgcggtgc	ss
855	sszgcagctgcggtgcggaagaa	ss
856	sszgcagctgcggtgcggaagaa	ss
857	gszgcagcagctgcggaagaa	ss
858	cszgcggtgcagctgcagcgagctgc	ss
859	tszgcggtgcagctgcagcgagctgc	o
860	gszgcagctgcagctgcagcgagctgc	o
861	tszgcggtgcagctgcagcgagctgc	o
862	tszgcggtgcagctgcagcgagctgc	ss
863	sszgcagctgcagctgcagcgagctgc	ss
864	tszgcagcagctgcagcgagctgc	o
865	tszgcagcagctgcagcgagctgc	o
866	tszgcagcagctgcagcgagctgc	o
867	tsztcctgcctgcctgc	o
868	tsztcctgcctgcctgc	ss
869	cszgcagctgcagctgcagcgagctgc	ss
870	tszgcagctgcagctgcagcgagctgc	ss
871	tszgcagctgcagctgcagcgagctgc	ss
872	cszgcagctgcagctgcagcgagctgc	ss
873	tszgcagctgcagctgcagcgagctgc	ss
874	sszgcagctgcagctgcagcgagctgc	ss
875	tszgcagctgcagctgcagcgagctgc	ss
876	tszgcagctgcagctgcagcgagctgc	ss
877	cszgcagctgcagctgcagcgagctgc	ss
878	cszgcagctgcagctgcagcgagctgc	ss
879	cszgcagctgcagctgcagcgagctgc	ss
880	cszgcagctgcagctgcagcgagctgc	ss
881	gszgcagctgcagctgcagcgagctgc	ss
882	tszgcagctgcagctgcagcgagctgc	ss
883	tszgcagctgcagctgcagcgagctgc	ss
884	tszgcagctgcagctgcagcgagctgc	ss
885	tszgcagctgcagctgcagcgagctgc	ss
886	tszgcagctgcagctgcagcgagctgc	ss
887	tszgcagctgcagctgcagcgagctgc	ss
888	tszgcagctgcagctgcagcgagctgc	ss
889	tszgcagctgcagctgcagcgagctgc	ss
890	tszgcagctgcagctgcagcgagctgc	ss
891	tszgcagctgcagctgcagcgagctgc	ss
892	tszgcagctgcagctgcagcgagctgc	ss
893	tszgcagctgcagctgcagcgagctgc	ss

[illegible]

943	ltgpttttttttggtttttttttttgg	8
946	lttctctcttctctctcttttctctctc	8
947	gggttcactcgtctctctctctctctc	8
948	tcactctctctctctctctctctctct	808
949	tcactctctctctctctctctctctct	
950	tcactctctctctctctctctctctct	
951	tcactctctctctctctctctctctct	
952	tcactctctctctctctctctctctct	
953	tcactctctctctctctctctctctct	
954	tcactctctctctctctctctctctct	
955	tcactctctctctctctctctctctct	
956	tcactctctctctctctctctctctct	
957	tcactctctctctctctctctctctct	
958	tcactctctctctctctctctctctct	
959	gggtctctctctctctctctctctct	808
960	gggtctctctctctctctctctctct	808
961	tttttttttttttttttttttttttttt	808
962	tttttttttttttttttttttttttttt	po
963	tttttttttttttttttttttttttttt	po
964	tcactctctctctctctctctctctct	po
965	tcactctctctctctctctctctctct	
966	tcactctctctctctctctctctctct	
967	tcactctctctctctctctctctctct	
968	gggtctctctctctctctctctctct	808
969	gggtctctctctctctctctctctct	808
970	gggtctctctctctctctctctctct	808
971	tcactctctctctctctctctctctct	
972	gggtctctctctctctctctctctct	808
973	gggtctctctctctctctctctctct	808
974	gggtctctctctctctctctctctct	808
975	gggtctctctctctctctctctctct	808
976	gggtctctctctctctctctctctct	808
977	gggtctctctctctctctctctctct	808
978	gggtctctctctctctctctctctct	808
979	gggtctctctctctctctctctctct	808
980	gggtctctctctctctctctctctct	808
981	gggtctctctctctctctctctctct	808
982	tcactctctctctctctctctctctct	808
983	tcactctctctctctctctctctctct	808
984	tcactctctctctctctctctctctct	808
985	tcactctctctctctctctctctctct	808
986	gggtctctctctctctctctctctct	808
987	gggtctctctctctctctctctctct	808
988	gggtctctctctctctctctctctct	808
989	gggtctctctctctctctctctctct	808
990	gggtctctctctctctctctctctct	808
991	gggtctctctctctctctctctctct	808
992	tcactctctctctctctctctctctct	808
993	tcactctctctctctctctctctctct	808
994	tcactctctctctctctctctctctct	808
995	tcactctctctctctctctctctctct	808
996	tcactctctctctctctctctctctct	808
997	tcactctctctctctctctctctctct	808
998	tcactctctctctctctctctctctct	808
999	tcactctctctctctctctctctctct	808
1000	tcactctctctctctctctctctctct	808

999	ggggggagcagctcgggggggg	gss
996	gggggaaagctcgggggggg	gss
997	ggg ggg aag atc ggc ggg ggg	gss
996	ggg ggg cga tgg tgg ggg ggg	gss
999	aag gac gtt aag	gss
1000	aagagcttaaa	gss
1001	aagagcttaaa	gss
1002	aagcttcgaaaa	gss
1003	gggggctcagctcgggggg	gss
1004	ggggggcagagcggggggg	gss
1005	gggtgcttaataaagc	gss
1006	gggtccttggtgcttct	gss
1007	ccattccattcgttgtaac	gss
1008	tatgcttatcagtgatga	gss
1009	agcctacgctctcaaccca	gss
1010	ttcctggaactaccttctga	gss
1011	atagagggctcaaacggt	gss
1012	tttaacccggtcagcgggt	gss
1013	cttaacccgagctcaagctg	gss
1014	cctgagattgattggtttag	gss
1015	tataagcctcgctcgaatg	gss
1016	cctgtcgagcgagagcttata	gss
1017	tgggtggtgggggagagcttc	gss
1018	gggctcctccacccacacac	gss
1019	gggcttcgacttcggtgggg	gss
1020	tgggactctcttgcgcgt	gss
1021	atggtctgtcgccagcgata	gss
1022	accgagatcaggcggaagaga	gss
1023	tcocattcggttcgagcgtt	gss
1024	gggaagaaacccagctcgttgg	gss
1025	aacacagagccagcggggga	gss
1026	gggctcggaacttcggggaag	gss
1027	tgggttaaccttcaggggggt	gss
1028	ctgggtgcctcagcaagat	gss
1029	gggactccatcaccagagat	gss
1030	cccgaaaggaagacccagcgt	gss
1031	ctgtggtctatgctataaag	gss
1032	aattacgcttaaggtatggg	gss
1033	aaactcggtctactactcagg	gss
1034	ataagctcattttgggaactac	gss
1035	cccaatccatcactagctaat	gss
1036	gggggtcgttcgagcgggggg	gss
1037	gggggttcgagcgggggggg	gss
1038	gggggaagcttcgaaagggggg	gss
1039	tccctggcggggaact	gss
1040	ggggagcagcagctcgttgggggg	gss
1041	gggggaagcagctcggggggg	gss
1042	gggggaagcagctcgggttggggg	gss
1043	gggggaagcagctcgggttggg	gss

1044	gggttcgacgtacgtcgcggggggg	gag
1045	gggttcgacgtacgtcgcggggg	gag
1046	gggttcgacgtacgtcgcggggg	gag
1047	gggttcgacgtacgtcgcggggg	gag
1048	gggttcgacgtacgtcgcggggg	gag
1049	gggttcgacgtacgtcgcggggg	gag
1050	gggttcgacgtacgtcgcggggg	gag
1051	gggttcgacgtacgtcgcggggg	gag
1052	gggttcgacgtacgtcgcggggg	gag
1053	gggttcgacgtacgtcgcggggg	gag
1054	gggttcgacgtacgtcgcggggg	gag
1055	gggttcgacgtacgtcgcggggg	gag
1056	gggttcgacgtacgtcgcggggg	gag
1057	gggttcgacgtacgtcgcggggg	gag
1058	gggttcgacgtacgtcgcggggg	gag
1059	gggttcgacgtacgtcgcggggg	gag
1060	gggttcgacgtacgtcgcggggg	gag
1061	gggttcgacgtacgtcgcggggg	gag
1062	gggttcgacgtacgtcgcggggg	gag
1063	gggttcgacgtacgtcgcggggg	gag
1064	gggttcgacgtacgtcgcggggg	gag
1065	gggttcgacgtacgtcgcggggg	gag
1066	gggttcgacgtacgtcgcggggg	gag
1067	gggttcgacgtacgtcgcggggg	gag
1068	gggttcgacgtacgtcgcggggg	gag
1069	gggttcgacgtacgtcgcggggg	gag
1070	gggttcgacgtacgtcgcggggg	gag
1071	gggttcgacgtacgtcgcggggg	gag
1072	gggttcgacgtacgtcgcggggg	gag
1073	gggttcgacgtacgtcgcggggg	gag
1074	gggttcgacgtacgtcgcggggg	gag
1075	gggttcgacgtacgtcgcggggg	gag
1076	gggttcgacgtacgtcgcggggg	gag
1077	gggttcgacgtacgtcgcggggg	gag
1078	gggttcgacgtacgtcgcggggg	gag
1079	gggttcgacgtacgtcgcggggg	gag
1080	gggttcgacgtacgtcgcggggg	gag
1081	gggttcgacgtacgtcgcggggg	gag
1082	gggttcgacgtacgtcgcggggg	gag
1083	gggttcgacgtacgtcgcggggg	gag
1084	gggttcgacgtacgtcgcggggg	gag
1085	gggttcgacgtacgtcgcggggg	gag
1086	gggttcgacgtacgtcgcggggg	gag
1087	gggttcgacgtacgtcgcggggg	gag
1088	gggttcgacgtacgtcgcggggg	gag
1089	gggttcgacgtacgtcgcggggg	gag
1090	gggttcgacgtacgtcgcggggg	gag
1091	gggttcgacgtacgtcgcggggg	gag
1092	gggttcgacgtacgtcgcggggg	gag

1093	gggttcgacgtacgtcgcggggg	gag
1094	gggttcgacgtacgtcgcggggg	gag
1095	gggttcgacgtacgtcgcggggg	gag
1096	gggttcgacgtacgtcgcggggg	gag
1097	gggttcgacgtacgtcgcggggg	gag

While CpG effects in mice are well characterized, information regarding the human system is limited. CpG phosphorothioate oligonucleotides with strong stimulatory activity in the mouse system show lower activity on human and other non-rodent immune cells. In the examples the development of a potent human CpG motif and the characterization of its effects and mechanisms of action on human primary B-cells is described. DNA containing this CpG motif strongly stimulated primary human B-cells to proliferate, to produce IL-6 and to express increased levels of CD86, CD40, CD54 and MHC II. It increased DNA binding activity of the transcription factors NFkB and AP-1, as well as phosphorylation of the stress activated protein kinases JNK and p38, and the transcription factor ATF-2. B-cell signalling pathways activated by CpG DNA were different from those activated by the B-cell receptor which activated ERK and a different isoform of JNK, but did not activate p38 and ATF-2. In general the data on CpG DNA-initiated signal transduction are consistent with those obtained in mice (Hoeker H, et al. 1998. *Embo J* 17:6230, Yi A. K., and Kleeg A. M. 1998. *J Immunol* 161:4493).

The preferred non-rodent motif is 5' TCGTCGTT 3'. Base exchanges within the most potent flanking CpG motif (5' TCGTCGTT 3') diminished the activity of the oligonucleotide. The thymidines at the 5' and the 3' position of this motif were more important than the thymidine at the middle position. An adenine or guanosine at the middle position produced a decrease in the activity.

Of note, our studies demonstrate that one human CpG motif within a phosphorothioate oligonucleotide (2080) is sufficient to produce the maximal effect, and that additional CpG motifs (2059) did not further enhance the activity. The oligonucleotide with the 8mer motif 5' TCG TCG TT 3' (2080) containing two CpG dinucleotides showed the highest activity in the studies. Replacement of the bases flanking the two CpG dinucleotides (5' position, middle position, 3' position) reduced the activity of this sequence. Both CpG dinucleotides within the 8mer CpG motif were required for the optimal activity (2108, 2106). Cytidine methylation of the CpG dinucleotides (2095) abolished the activity of 2080, while methylation of an unrelated

cytidine (2094) did not. The addition of two CpG motifs into the sequence of 2080 resulting in 2039 did not further increase the activity of the phosphodiester oligonucleotide. The sequence of 2080 with a phosphorothioate backbone (2116) demonstrated less activity, suggesting that additional CpG motifs are preferred for a potent phosphorothioate oligonucleotide.

It has been discovered according to the invention that the immunostimulatory nucleic acids have dramatic immune stimulatory effects on human cells such as NK cells, B cells, and DCs *in vitro*. It has been demonstrated that that the *in vitro* assays used herein predict *in vivo* effectiveness as a vaccine adjuvant in non-ratent vertebrates (Example 12), suggesting that immunostimulatory nucleic acids are effective therapeutic agents for human vaccination, cancer immunotherapy, asthma immunotherapy, general enhancement of immune function, enhancement of hematopoietic recovery following radiation or chemotherapy, and other immune modulatory applications.

Thus the immunostimulatory nucleic acids are useful in some aspects of the invention as a prophylactic vaccine for the treatment of a subject at risk of developing an infection with an infectious organism or a cancer in which a specific cancer antigen has been identified or an allergy or asthma where the allergen or predisposition to asthma is known. The immunostimulatory nucleic acids can also be given without the antigen or allergen for shorter term protection against infection, allergy or cancer, and in this case repeated doses will allow longer term protection. A subject at risk as used herein is a subject who has any risk of exposure to an infection causing pathogen or a cancer or an allergen or a risk of developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent is found or it may be a subject who through lifestyle or medical procedures is exposed to bodily fluids which may contain infectious organisms or directly to the organism or even any subject living in an area where an infectious organism or an allergen has been identified. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject may be exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. A subject at risk of developing an allergy to asthma includes those subjects that have been identified as having an allergy or

asthma but that don't have the active disease during the immunostimulatory nucleic acid treatment as well as subjects that are considered to be at risk of developing these diseases because of genetic or environmental factors.

A subject at risk of developing a cancer is one who is who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins, or a subject who has previously been treated for cancer and is in apparent remission. When a subject at risk of developing a cancer is treated with an antigen specific for the type of cancer to which the subject is at risk of developing and a immunostimulatory nucleic acid, the subject may be able to kill the cancer cells as they develop. If a tumor begins to form in the subject, the subject will develop a specific immune response against the tumor antigen.

In addition to the use of the immunostimulatory nucleic acids for prophylactic treatment, the invention also encompasses the use of the immunostimulatory nucleic acids for the treatment of a subject having an infection, an allergy, asthma, or a cancer. A subject having an infection is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The immunostimulatory nucleic acids can be used with an antigen to mount an antigen specific systemic or mucosal immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. It is particularly important to develop effective vaccine strategies and treatments to protect the body's mucosal surfaces, which are the primary site of pathogenic entry.

A subject having an allergy is a subject that has or is at risk of developing an allergic reaction in response to an allergen. An allergy refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or hay fever, conjunctivitis, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.

Currently, allergic diseases are generally treated by the injection of small doses of antigen followed by subsequent increasing dosage of antigen. It is believed that this procedure induces toleration to the allergen to prevent further allergic reactions. These

methods, however, can take several years to be effective and are associated with the risk of side effects such as anaphylactic shock. The methods of the invention avoid these problems.

Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by systemic or mucosal administration of immunostimulatory nucleic acids are predominantly of a class called Th1 (examples are IL-12 and IFN- γ) and these induce both humoral and cellular immune responses. The types of antibodies associated with a Th1 response are generally more protective because they have high neutralization and opsonization capabilities. The other major type of immune response, which is associated with the production of IL-4, IL-5 and IL-10 cytokines, is termed a Th2 immune response. Th2 responses involve predominantly antibodies and these have less protective effect against infection and some Th2 isotypes (e.g., IgE) are associated with allergy. In general, it appears that allergic diseases are mediated by Th2 type immune responses while Th1 responses provide the best protection against infection, although excessive Th1 responses are associated with autoimmune diseases. Based on the ability of the immunostimulatory nucleic acids to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose for inducing an immune response of a immunostimulatory nucleic acid can be administered to a subject to treat or prevent an allergy.

Thus, the immunostimulatory nucleic acids have significant therapeutic utility in the treatment of allergic and non-allergic conditions such as asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN- γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. Asthma refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

A subject having a cancer is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are

not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intracerebral neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; renal cancer, as well as other carcinomas and sarcomas. In one embodiment the cancer is hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, or colon carcinoma.

A subject according to the invention is a non-rodent subject. A non-rodent subject shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, e.g., monkey, and fish (aquaculture species), e.g. salmon, but specifically excluding rodents such as rats and mice.

Thus, the invention can also be used to treat cancer and tumors in non human subjects. Cancer is one of the leading causes of death in companion animals (i.e., cats and dogs). Cancer usually strikes older animals which, in the case of house pets, have become integrated into the family. Forty-five % of dogs older than 10 years of age, are likely to succumb to the disease. The most common treatment options include surgery, chemotherapy and radiation therapy. Others treatment modalities which have been used with some success are laser therapy, cryotherapy, hyperthermia and immunotherapy.

The choice of treatment depends on type of cancer and degree of dissemination. Unless the malignant growth is confined to a discrete area in the body, it is difficult to remove only malignant tissue without also affecting normal cells.

Malignant disorders commonly diagnosed in dogs and cats include but are not limited to lymphosarcoma, osteosarcoma, mammary tumors, mastocytoma, brain tumor, melanoma, adenocarcinoma carcinoma, carcinoid lung tumor, bronchial gland tumor, bronchiolar adenocarcinoma, fibroma, myxochondroma, pulmonary sarcoma, neurosarcoma, osteoma, papilloma, retinoblastoma, Ewing's sarcoma, Wilms' tumor, Burkitt's lymphoma, microglioma, neuroblastoma, osteoclastoma, oral neoplasia, fibrosarcoma, osteosarcoma and rhabdomyosarcoma. Other neoplasias in dogs include genital squamous cell carcinoma, transmissible venereal tumor, testicular tumor,

semimoma, Sertoli cell tumor, hemangioepithelioma, histiocytoma, ellipoma (granulocytic sarcoma), corneal papilloma, corneal squamous cell carcinoma, hemangiosarcoma, pleural mesothelioma, basal cell tumor, thymoma, stomach tumor, adrenal gland carcinoma, oral papillomatosis, hemangioendothelioma and cytoductoma.

Additional malignancies diagnosed in cats include follicular lymphoma, intestinal lymphosarcoma, fibrosarcoma and pulmonary squamous cell carcinoma. The feral, an ever-more popular house pet is known to develop insulinoma, lymphoma, sarcoma, neuroma, pancreatic islet cell tumor, gastric MALT lymphoma and gastric adenocarcinoma.

Neoplasias affecting agricultural livestock include leukemias, hemangioepithelioma and bovine ocular neoplasia (in cattle), preputial fibrosarcoma, ulcerative squamous cell carcinoma, preputial carcinoma, connective tissue neoplasia and mastocytoma (in horses); hepatocellular carcinoma (in swine); lymphoma and pulmonary adenomatosis (in sheep); pulmonary sarcoma, lymphoma, Rous sarcoma, reticulendotheliosis, fibrosarcoma, epithelioma, B-cell lymphoma and lymphoid leukemia (in avian species); retinoblastoma, hepatic neoplasia, lymphosarcoma (lymphoblastic lymphoma), plasmacytoid leukemia and swimbladder sarcoma (in fish), caecum lymphadenitis (C.A.): diarrhea, infection, contagious disease of sheep and goats caused by the bacterium *Corynebacterium pseudotuberculosis*, and contagious lung tumor of sheep caused by *Jasgidiella*.

The subject is exposed to the antigen. As used herein, the term exposed to refers to either the active step of contacting the subject with an antigen or the passive exposure of the subject to the antigen *in vivo*. Methods for the active exposure of a subject to an antigen are well-known in the art. In general, an antigen is administered directly to the subject by any means such as intravenous, intramuscular, oral, transdermal, mucosal, intranasal, intratracheal, or subcutaneous administration. The antigen can be administered systemically or locally. Methods for administering the antigen and the immunostimulatory nucleic acid are described in more detail below. A subject is passively exposed to an antigen if an antigen becomes available for exposure to the immune cells in the body. A subject may be passively exposed to an antigen, for instance, by entry of a foreign pathogen into the body or by the development of a tumor cell expressing a foreign antigen on its surface.

The methods in which a subject is passively exposed to an antigen can be particularly dependent on timing of administration of the immunostimulatory nucleic acid. For instance, in a subject at risk of developing a cancer or an infectious disease or an allergic or asthmatic response, the subject may be administered the immunostimulatory nucleic acid on a regular basis when that risk is greatest, i.e., during allergy season or after exposure to a cancer causing agent. Additionally the immunostimulatory nucleic acid may be administered to travelers before they travel to foreign lands where they are at risk of exposure to infectious agents. Likewise the immunostimulatory nucleic acid may be administered to soldiers or civilians at risk of exposure to bio warfare to induce a systemic or mucosal immune response to the antigen when and if the subject is exposed to it.

An antigen as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, glycolipids, carbohydrates, viruses and viral extracts and multicellular organisms such as parasites and allergens. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens, and allergens.

A cancer antigen as used herein is a compound, such as a peptide or protein, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 1994, *Cancer Research*, 54:1035, by partially purifying the antigens, by recombinant technology, or by *de novo* synthesis of known antigens. Cancer antigens include but are not limited to antigens that are recombinantly expressed, an immunogenic portion of, or a whole tumor or cancer. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

A microbial antigen as used herein is an antigen of a microorganism and includes but is not limited to virus, bacteria, parasites, and fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also

synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to those of ordinary skill in the art.

Examples of viruses that have been found in humans include but are not limited to: *Rotoviridae* (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-1-F; *Picornaviridae* (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calchiviridae* (e.g. strains that cause gastroenteritis); *Togaviridae* (e.g. equine encephalitis viruses, rubella viruses); *Flaviviridae* (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g. coronavirus); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Coronaviridae* (e.g. coronavirus); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Flaviviridae* (e.g. ebola viruses); *Paramyxoviridae* (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g. influenza viruses); *Bunyaviridae* (e.g. Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); *Arna viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g. reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; *Poxviridae* (variola virus, vaccinia virus, pox virus); and *Iridoviridae* (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to, *Pasteurella* species, *Staphylococci* species, and *Streptococci* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to, *Helicobacter*

plyoritis, *Borella burgdorferi*, *Legionella pneumophila*, *Mycobacterium* spp (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. goodii*), *Staphylococcus aureus*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic spp.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* spp., *Enterococcus* spp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* spp., *Dysphelobacterium*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* spp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenax*, *Leptospira*, *Rickettsia*, and *Achromyces israelii*.

Examples of fungi include *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomycosis dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*.

Other infectious organisms (i.e., protists) include *Plasmodium* spp. such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax* and *Toxoplasma gondii*. Blood-borne and/or tissue parasites include *Plasmodium* spp., *Babesia microti*, *Babesia divergens*, *Leishmania tropica*, *Leishmania* spp., *Leishmania braziliensis*, *Leishmania donovani*, *Trypanosoma gambiense* and *Trypanosoma rhodesiense* (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Toxoplasma gondii*.

Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A. Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

Although many of the microbial antigens described above relate to human disorders, the invention is also useful for treating other nonhuman vertebrates. Nonhuman vertebrates are also capable of developing infections which can be prevented or treated with the immunostimulatory nucleic acids disclosed herein. For instance, in addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals.

As used herein, the term treat, treated, or treating when used with respect to an infectious disease refers to a prophylactic treatment which increases the resistance of a subject (a subject at risk of infection) to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen as well as a treatment after the subject (a subject who has been infected) has become infected in order to fight the infection, e.g., reduce or eliminate the infection or prevent it from becoming worse.

Many vaccines for the treatment of non-human vertebrates are disclosed in Bennett, K. *Compendium of Veterinary Products*, 3rd ed. North American

Compendium, Inc., 1995. As discussed above, antigens include infectious microbes such as virus, parasite, bacteria and fungi and fragments thereof, derived from natural sources or synthetically. Infectious viruses of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. This group of retroviruses includes both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroup of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV)) and C-type group B (including feline leukemia virus (FeLV), gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (REV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, STV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of other RNA viruses that are antigens in vertebrate animals include, but are not limited to, members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Bagdadungu virus, Kemerovo virus, African horse sickness

virus, and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picornaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A virus, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus smita, Bovine enteroviruses, Porcine enteroviruses, the genus Cardiovirus (Encephalomyocarditis virus (EMCV), Mengovirus), the genus Rhinovirus (human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Astrovirus (Foot and Mouth disease (FMDV); the family Caliciviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Bassett equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Onk encephalitis virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukerivirus (Uukerani and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; Influenza type B (many human subtypes), and Influenza type C (possible separate genus); the family Paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Poxvirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pseudomonia virus); the family Rhabdoviridae, including the genus Vesiculovirus (VSV), Chandelipya virus, Pinder-Hart Park virus), the genus

Lyssavirus (Rabies virus), fish Rhabdoviruses, and two probable Rhabdoviruses (Marburg virus and Ebola virus), the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family Coronaviridae, including Infectious Bronchitis Virus (IBV), Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that are antigens in vertebrate animals include, but are not limited to, the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalo pox, Rabbit pox, Ectromelia), the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowl pox, other avian poxvirus), the genus Capripoxvirus (sheep pox, goat pox), the genus Suipoxvirus (Swine pox), the genus Parapoxvirus (contagious pustular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine and monkeys); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes salmوني, Herpesvirus steles, Herpesvirus lyMylagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A,B,C,D,E and ungrouped; alkanine adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RVK), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotropic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and

Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

Each of the foregoing lists is illustrative, and is not intended to be limiting.

In addition to the use of the immunostimulatory nucleic acids to induce an antigen specific immune response in humans, the methods of the preferred embodiments are particularly well suited for treatment of birds such as hens, chickens, turkeys, ducks, geese, quail, and pheasant. Birds are prime targets for many types of infections.

Hatchling birds are exposed to pathogenic microorganisms shortly after birth. Although these birds are initially protected against pathogens by maternal derived antibodies, this protection is only temporary, and the bird's own immature immune system must begin to protect the bird against the pathogens. It is often desirable to prevent infection in young birds when they are most susceptible. It is also desirable to prevent against infection in older birds, especially when the birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the immunostimulatory nucleic acid and the non-nucleic acid adjuvant of the invention to birds to enhance an antigen-specific immune response when antigen is present.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yusaka et al., 1979, Avian Dis. 23:366-385). Since that time, CIAV has been detected in commercial poultry in all major poultry producing countries (van Bulow et al., 1991, pp. 690-699) in Diseases of Poultry, 9th edition, Iowa State University Press).

CIAV infection results in a clinical disease, characterized by anemia, hemorrhage and immunosuppression, in young susceptible chickens. Atrophy of the thymus and of the bone marrow and consistent lesions of CIAV-infected chickens are also characteristic of CIAV infection. Lymphocyte depletion in the thymus, and occasionally in the bursa of Fabricius, results in immunosuppression and increased susceptibility to secondary viral, bacterial, or fungal infections which then complicate the course of the disease. The immunosuppression may cause aggravated disease after infection with one or more of Marek's disease virus (MDV), infectious bursal disease virus, reticuloendotheliosis virus, adenovirus, or reovirus. It has been reported that pathogenesis of MDV is enhanced by CIAV (DeBoer et al., 1989, p. 28 In Proceedings

- 73 -

of the 34th Western Poultry Diseases Conference, Tampa, Aziz.). Further, it has been reported that CLAV agglutinates the signs of infectious bursal disease (Rosenberger et al., 1989, Avian Dis. 33:707-713). Chickens develop an age resistance to experimentally induced disease due to CAA. This is essentially completed by the age of 2 weeks, but older birds are still susceptible to infection (Yusa, N. et al., 1979 *supra*; Yusa, N. et al., Avian Diseases 24, 202-209, 1980). However, if chickens are dually infected with CAA and an immunosuppressive agent (IBDV, MDV etc.), age resistance against the disease is delayed (Yusa, N. et al., 1979 and 1980 *supra*; Bulow von V. et al., J. Veterinary Medicine 33, 93-116, 1986). Characteristics of CLAV that may potentiate disease transmission include high resistance to environmental inactivation and some common disinfectants. The economic impact of CLAV infection on the poultry industry is clear from the fact that 10% to 30% of infected birds in disease outbreaks die.

Vaccination of birds, like other vertebrate animals can be performed at any age. Normally, vaccinations are performed at up to 12 weeks of age for a live microorganism and between 14-18 weeks for an inactivated microorganism or other type of vaccine. For in ovo vaccination, vaccination can be performed in the last quarter of embryo development. The vaccine may be administered subcutaneously, by spray, orally, intracocularly, intratracheally, nasally, or by other mucosal delivery methods described herein. Thus, the immunostimulatory nucleic acids of the invention can be administered to birds and other non-human vertebrates using routine vaccination schedules and the antigen can be administered after an appropriate time period as described herein.

Cattle and livestock are also susceptible to infection. Diseases which affect these animals can produce severe economic losses, especially amongst cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats.

Cows can be infected by bovine viruses. Bovine viral diarrhoea virus (BVDV) is a small enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although, Pestiviruses were previously classified in the Toga viridae family, some studies have suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups (Trnacci, et al., 1991).

- 74 -

BVDV, which is an important pathogen of cattle can be distinguished, based on cell culture analysis, into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes. The NCP biotype is more widespread although both biotypes can be found in cattle. If a pregnant cow becomes infected with an NCP strain, the cow can give birth to a persistently infected and specifically immunotolerant calf that will spread virus during its lifetime. The persistently infected cattle can succumb to mucosal disease and both biotypes can then be isolated from the animal. Clinical manifestations can include abortion, leucopenia, and respiratory problems, mucosal disease and mild diarrhea. In addition, severe thrombocytopenia, associated with herd epidemics, that may result in the death of the animal has been described and strains associated with this disease occur more virulent than the classical BVDV.

Equine herpes viruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal diseases. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and central nervous system disorders. Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts for 8 to 10 days. Immunologically experienced mares may be re-infected via the respiratory tract without disease becoming apparent, so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of either sex at any age, leading to lack of co-ordination, weakness and posterior paralysis (Telford, E. A. R. et al., Virology 189, 304-316, 1992). Other EHV's include EHV-2, or equine cytomegalovirus, EHV-3, equine colic exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

Sheep and goats can be infected by a variety of dangerous microorganisms including virus-mech.

Primates such as monkeys, apes and managoes can be infected by simian immunodeficiency virus. Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in managoes (Stott et al. (1990) Lancet 36:1538-1541; Desrochers et al. PNAS USA (1989) 86:6353-6357; Murphy-Corb et al. (1989) Science 246:1293-1297, and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV gp120 vaccine has been

reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

Cats, both domestic and wild, are susceptible to infection with a variety of microorganisms. For instance, feline infectious peritonitis is a disease which occurs in both domestic and wild cats, such as lions, leopards, cheetahs, and jaguars. When it is desirable to prevent infection with this and other types of pathogenic organisms in cats, the methods of the invention can be used to vaccinate cats to protect them against infection.

Domestic cats may become infected with several retroviruses, including but not limited to feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C coronavirus (RD-114), and feline syncytium-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemia, immune mediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

The discovery of feline T-lymphotropic lentivirus (also referred to as feline immunodeficiency) was first reported in Pedersen et al. (1987) Science 235:790-793. Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258; and Adley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. USA 86:2448-2452 and 86:4355-4360.

Feline infectious peritonitis (FIP) is a sporadic disease occurring unpredictably in domestic and wild Felidae. While FIP is primarily a disease of domestic cats, it has been diagnosed in lions, mountain lions, leopards, cheetahs, and the jaguar. Smaller wild cats that have been afflicted with FIP include the lynx and caracal, sand cat, and pallas cat. In domestic cats, the disease occurs predominantly in young animals, although cats of all ages are susceptible. A peak incidence occurs between 6 and 12 months of age. A decline in incidence is noted from 5 to 13 years of age, followed by an increased incidence in cats 14 to 15 years old.

Viral, bacterial, and parasitic diseases in fin-fish, shellfish or other aquatic life forms pose a serious problem for the aquaculture industry. Owing to the high density of animals in the hatchery tanks or enclosed marine farming areas, infectious diseases may eradicate a large proportion of the stock in, for example, a fin-fish, shellfish, or other aquatic life forms facility. Prevention of disease is a more desired remedy to these threats to fish than intervention once the disease is in progress. Vaccination of fish is the only preventative method which may offer long-term protection through immunity. Nucleic acid based vaccinations are described in US Patent No. 5,780,448 issued to Davis.

The fish immune system has many features similar to the mammalian immune system, such as the presence of B cells, T cells, lymphokines, complement, and immunoglobulins. Fish have lymphocyte subclasses with roles that appear similar in many respects to those of the B and T cells of mammals. Vaccines can be administered by immersion or orally.

Aquaculture species include but are not limited to fin-fish, shellfish, and other aquatic animals. Fin-fish include all vertebrate fish, which may be bony or cartilaginous fish, such as, for example, salmonids, carp, catfish, yellowtail, sea bass, and sea bass. Salmonids are a family of fin-fish which include trout (including rainbow trout), salmon, and Arctic char. Examples of shellfish include, but are not limited to, clams, lobster, shrimp, crab, and oysters. Other cultured aquatic animals include, but are not limited to, cells, squid, and octopi.

Polypeptides of viral aquaculture pathogens include but are not limited to glycoprotein (G) or nucleoprotein (N) of viral hemorrhagic septicemia virus (VHSV); G or N proteins of infectious hematopoietic necrosis virus (IHNV); VP1, VP2, VP3 or N structural proteins of infectious pancreatic necrosis virus (IPNV); G protein of spring viremia of carp (SVC); and a membrane-associated protein, tegument or capsid protein or glycoprotein of channel catfish virus (CCV).

Typical parasites infecting horses are *Gasterophilus* spp.; *Elmiera leuckartii*, *Giardia* spp.; *Trichostrongylus axei*; *Babesia* spp. (RBC's); *Theileria equi*; *Trypanosoma* spp.; *Clasidiella equi*; *Sarcocystis* spp.

- 77 -

Typical parasites infecting swine include *Eimeria babielacki*, *Eimeria acabra*, *Isospora suis*, *Giardia* spp.; *Belontidium coli*, *Eximamoeba histolytica*, *Toxoplasma gondii* and *Sarcocystis* spp., and *Trichinella spiralis*.

The major parasites of dairy and beef cattle include *Eimeria* spp.

- 5 *Cryptosporidium* sp., *Giardia* spp.; *Toxoplasma gondii*, *Babesia bovis* (RBC), *Babesia biguttata* (RBC), *Trypanosoma* spp. (glasma), *Theileria* spp. (RBC), *Theileria parva* (lymphocystis), *Trichostrongylus axei*, and *Sarcocystis* spp.

- 10 The major parasites of ruminants include *Trichostrongylus axei*, *Coccidia* (*Eimeria* spp.), *Plasmodium relictum*, *Leucocytozon danielwasyi* (ovine), *Haemonchus* spp., *Trypanosoma* spp.; *Histomonas*, *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, *Giardia*, *Eimeria*, *Toxoplasma*.

Typical parasites infecting sheep and goats include *Eimeria* spp.,

- 15 *Cryptosporidium* sp., *Giardia* sp.; *Toxoplasma gondii*, *Babesia* spp. (RBC), *Trypanosoma* spp. (glasma), *Theileria* spp. (RBC), and *Sarcocystis* spp.

- 20 Typical parasitic infections in poultry include coccidiosis caused by *Eimeria acervulina*, *E. necatrix*, *E. tenella*, *Isospora* spp. and *Eimeria brunetti*; histomoniasis, caused by *Histomonas meleagridis* and *Histomonas gallinarum*; trichomoniasis caused by *Trichomonas gallinarum*; and hexamitiasis caused by *Hexamita meleagridis*. Poultry can also be infected *Eimeria maxima*, *Eimeria meleagridis*, *Eimeria adenoviridis*, *Eimeria meleagridis*, *Cryptosporidium*, *Eimeria brunetti*, *Eimeria adenoviridis*, *Leucocytozon* spp., *Plasmodium* spp., *Haemonchus meleagridis*, *Toxoplasma gondii* and *Sarcocystis*.

The methods of the invention can also be applied to the treatment and/or

prevention of parasitic infection in dogs, cats, birds, fish and fawns. Typical parasites of birds include *Trichostrongylus gallinarum*, *Eimeria* spp., *Isospora* spp., *Giardia*, *Cryptosporidium*, *Sarcocystis* spp., *Toxoplasma gondii*.

- 25 *Haemonchus/parahaeemonchus*, *Plasmodium* spp., *Leucocytozon/Albia*,

- Acanthamoeba*, *Trypanosoma* spp. Typical parasites infecting dogs include *Trichinella spiralis*, *Isospora* spp., *Sarcocystis* spp., *Cryptosporidium* spp., *Hammondi* spp., *Giardia duodenalis* (canine), *Belontidium coli*, *Eximamoeba histolytica*, *Hepatozoon canis*; *Toxoplasma gondii*, *Trypanosoma cruzi*, *Babesia canis*, *Leishmania amastigotes*; *Neospora caninum*.

- 78 -

Typical parasites infecting feline species include *Isospora* spp., *Toxoplasma gondii*, *Sarcocystis* spp., *Hammondi hammondi*, *Bemisia* spp., *Giardia* spp.; *Eximamoeba histolytica*, *Hepatozoon canis*, *Cyanozoon* sp., *Cyanozoon* sp., *Cyanozoon* sp. (red cells, RE cells).

5 Typical parasites infecting fish include *Hexamita* spp., *Eimeria* spp.; *Cryptobia* spp., *Noasema* spp., *Myxosoma* spp., *Chilodactylus* spp., *Trichodin* spp.; *Platyphora* spp., *Myxosoma hemerythra*, *Cordia* spp., *Ichthyophthirius* spp. and *Oodinium* spp.

- 10 Typical parasites of wild mammals include *Giardia* spp. (carnivores, herbivores), *Isospora* spp. (carnivores), *Eimeria* spp. (carnivores, herbivores), *Theileria* spp. (herbivores), *Babesia* spp. (carnivores, herbivores), *Trypanosoma* spp. (carnivores, herbivores), *Schistosoma* spp. (herbivores), *Fasciola hepatica* (herbivores), *Fascioloides magna* (herbivores), *Fasciola gigantica* (herbivores), *Trichinella spiralis* (carnivores, herbivores).

- 15 Parasitic infections in zoos can also pose serious problems. Typical parasites of the bovidae family (rhinoceros, antelope, bonting, eland, gnu, impala, kudu, giraffe) include *Eimeria* spp. Typical parasites in the primidae family (seal, sea lion) include *Eimeria phoca*. Typical parasites in the camelidae family (camels, llamas) include *Eimeria* spp. Typical parasites of the giraffidae family (giraffes) include *Eimeria* spp. Typical parasites in the elephantidae family (African and Asian) include *Fasciola* spp. Typical parasites of lower primates (chimpanzees, orangutans, apes, baboons, macaques, monkeys) include *Giardia* sp.; *Belontidium coli*, *Eximamoeba histolytica*, *Sarcocystis* spp., *Toxoplasma gondii*, *Plasmodium* spp. (RBC), *Babesia* spp. (RBC), *Trypanosoma* spp. (glasma), *Leishmania* spp. (macrophages).

- 20 Polyspecifics of bacterial pathogens include but are not limited to an iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of *Aeromonas salmonicida* which causes furunculosis, p57 protein of *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD), major surface associated antigen (msa), a surface expressed cytochrome (cyp), a surface expressed hemolysin (sh), and a flagellar antigen of *Yersinia enterocolitica*, an extracellular protein (ECP), an iron-regulated outer membrane protein (IROMP), and a structural protein of *Pasteurella*, an OMP and a flagellar protein of *Vibrio anguillarum* and *V. ordalii*, a flagellar protein, an OMP protein, *spoA*, and *purA* of *Edwardsiella ictaluri* and *E.*

- 25 iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of *Aeromonas salmonicida* which causes furunculosis, p57 protein of *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD), major surface associated antigen (msa), a surface expressed cytochrome (cyp), a surface expressed hemolysin (sh), and a flagellar antigen of *Yersinia enterocolitica*, an extracellular protein (ECP), an iron-regulated outer membrane protein (IROMP), and a structural protein of *Pasteurella*, an OMP and a flagellar protein of *Vibrio anguillarum* and *V. ordalii*, a flagellar protein, an OMP protein, *spoA*, and *purA* of *Edwardsiella ictaluri* and *E.*

- 30 iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of *Aeromonas salmonicida* which causes furunculosis, p57 protein of *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD), major surface associated antigen (msa), a surface expressed cytochrome (cyp), a surface expressed hemolysin (sh), and a flagellar antigen of *Yersinia enterocolitica*, an extracellular protein (ECP), an iron-regulated outer membrane protein (IROMP), and a structural protein of *Pasteurella*, an OMP and a flagellar protein of *Vibrio anguillarum* and *V. ordalii*, a flagellar protein, an OMP protein, *spoA*, and *purA* of *Edwardsiella ictaluri* and *E.*

tards, and surface antigen of *Ichthyophthirius*, and a structural and regulatory protein of *Cytophaga columba*; and a structural and regulatory protein of *Rickettsia*.

Polypeptides of a parasitic pathogen include but are not limited to the surface antigens of *Ichthyophthirius*.

- 3 An allergen refers to a substance (antigen) that can induce an allergic or autoimmune response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillins). Examples of natural, animal and plant allergens include but are not limited to proteins specific to the following genera: *Canine (Canis familiaris)*; *Dermatophagoides* (e.g. *Dermatophagoides farinae*); *Felis (Felis domesticus)*; *Ambrosia* (*Ambrosia artemisiifolia*; *Lolium* (e.g. *Lolium perenne* or *Lolium multiflorum*); *Cryptomeria* (*Cryptomeria japonica*); *Alternaria* (*Alternaria alternata*); *Alder*; *Alnus* (*Alnus gallica*); *Betula* (*Betula verrucosa*); *Quercus* (*Quercus alba*); *Olea* (*Olea europaea*); *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g. *Plantago lanceolata*); *Parietaria* (e.g. *Parietaria officinalis* or *Parietaria judaica*); *Blattella* (e.g. *Blattella germanica*); *Apis* (e.g. *Apis mellifera*); *Cypripedium* (e.g. *Cypripedium acaule*); *Cypripedium virginicum* and *Cypripedium macrocarpum*); *Juniperus* (e.g. *Juniperus communis*); *Juniperus virginiana*, *Juniperus communis* and *Juniperus aschaffi*; *Thuja* (e.g. *Thuja occidentalis*); *Chamaecyparis* (e.g. *Chamaecyparis obtusa*); *Periplaneta* (e.g. *Periplaneta americana*); *Agropyron* (e.g. *Agropyron repens*); *Secale* (e.g. *Secale cereale*); *Triticum* (e.g. *Triticum aestivum*); *Dactylis* (e.g. *Dactylis glomerata*); *Festuca* (e.g. *Festuca elatior*); *Poa* (e.g. *Poa pratensis* or *Poa compressa*); *Avena* (e.g. *Avena sativa*); *Holcus* (e.g. *Holcus lanatus*); *Anthoxanthum* (e.g. *Anthoxanthum odoratum*); *Arrhenatherum* (e.g. *Arrhenatherum elatius*); *Agrostis* (e.g. *Agrostis alba*); *Phleum* (e.g. *Phleum pratense*); *Phalaris* (e.g. *Phalaris arundinacea*); *Paspalum* (e.g. *Paspalum notatum*); *Sorghum* (e.g. *Sorghum halepense*); and *Bromus* (e.g. *Bromus tectorum*).

The antigen may be an antigen that is encoded by a nucleic acid vector or it may be not encoded in a nucleic acid vector. In the former case the nucleic acid vector is administered to the subject and the antigen is expressed *in vivo*. In the latter case the antigen may be administered directly to the subject. An antigen not encoded in a nucleic acid vector as used herein refers to any type of antigen that is not a nucleic acid. For instance, in some aspects of the invention the antigen not encoded in a nucleic acid

vector is a polypeptide. Minor modifications of the primary amino acid sequences of polypeptide antigens may also result in a polypeptide which has substantially equivalent antigenic activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as antigenicity still exists. The polypeptide may be, for example, a viral polypeptide.

The term substantially purified as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify viral or bacterial polypeptides using standard techniques for protein purification. The substantially pure polypeptide will often yield a single major band on a non-reducing polyacrylamide gel. In the case of partially glycosylated polypeptides or those that have several start codons, there may be several bands on a non-reducing polyacrylamide gel, but these will form a distinctive pattern for that polypeptide. The purity of the viral or bacterial polypeptide can also be determined by amino-terminal amino acid sequence analysis. Other types of antigens not encoded by a nucleic acid vector such as polysaccharides, small molecules, mimics etc are described above, and included within the invention.

The invention also utilizes polynucleotides encoding the antigenic polypeptides. It is envisioned that the antigen may be delivered to the subject in a nucleic acid molecule which encodes for the antigen such that the antigen must be expressed *in vivo*. Such antigens delivered to the subject in a nucleic acid vector are referred to as antigens encoded by a nucleic acid vector. The nucleic acid encoding the antigen is operatively linked to a gene expression sequence which directs the expression of the antigen nucleic acid within a eukaryotic cell. The gene expression sequence is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the antigen nucleic acid to which it is operatively linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPT), adenovirus deaminase, pyruvate kinase, b-actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from

the cytomegalovirus (CMV), simian virus (e.g., SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined antigen nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

The antigen nucleic acid is operatively linked to the gene expression sequence. As used herein, the antigen nucleic acid sequence and the gene expression sequence are said to be operably linked when they are covalently linked in such a way as to place the expression or transcription and/or translation of the antigen coding sequence under the influence or control of the gene expression sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the antigen sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the antigen sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to an antigen nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that antigen nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

The antigen nucleic acid of the invention may be delivered to the immune system alone or in association with a vector. In its broadest sense, a vector is any vehicle

capable of facilitating the transfer of the antigen nucleic acid to the cells of the immune system so that the antigen can be expressed and presented on the surface of the immune cell. The vector generally transports the nucleic acid to the immune cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. The vector optionally includes the above-described gene expression sequence to enhance expression of the antigen nucleic acid in immune cells. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagenda, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antigen nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to, nucleic acid sequences from the following viruses: retrovirus, such as Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kiegle, M., Gene Transfer and Expression, A Laboratory Manual W.H. Freeman C.O., New York (1990) and Murry, E.J. Methods in Molecular Biology, vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

A preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication -deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRCMV, SV40, and pBlueScript. Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA.

It has recently been discovered that genes carrying plasmids can be delivered to the immune system using bacteria. Modified forms of bacteria such as *Salmonella* can be transfected with the plasmid and used as delivery vehicles. The bacterial delivery vehicles can be administered to a host subject orally or by other administration means. The bacteria deliver the plasmid to immune cells, e.g. B cells, dendritic cells, likely by passing through the gut barrier. High levels of immune protection have been established using this methodology. Such methods of delivery are useful for the aspects of the

invention utilizing systemic delivery of antigen, immunostimulatory nucleic acid and/or other therapeutic agent.

Thus, the immunostimulatory nucleic acids are useful as vaccine adjuvants. It was previously established that CpG oligonucleotides are excellent vaccine adjuvants. It was also demonstrated, however, that CpG ODN which are superb vaccine adjuvants in mice are not the preferred adjuvants in non-rodent animals. In order to identify the best immunostimulatory nucleic acids for use as a vaccine adjuvant in humans and other non-rodent animals, *in vivo* screening of different nucleic acids for this purpose was conducted. Several *in vitro* assays were evaluated in mice for their predictive value of adjuvant activity *in vivo* in mice. During the course of this study, an *in vitro* test that is predictive of *in vivo* efficacy was identified. It was discovered, rather surprisingly, that both B cell and NK cell activation correlated particularly well with the ability of an immunostimulatory nucleic acid to enhance an *in vivo* immune response against an antigen.

The good predictive value of B cell activation for *in vivo* vaccine adjuvant activity is most likely linked to the central role of B cells in the establishment of a specific immune response. Polyclonal proliferation of B cells (induced by immunostimulatory nucleic acids) increases the likelihood of an antigen specific B cell/T helper cell match. Furthermore, enhanced expression of the co-stimulatory molecule CD86 on polyclonally expanded B cells activates antigen specific T helper cells. B cells also increase their CD40 expression in response to immunostimulatory nucleic acids improving the capability of CD40L expressing activated T helper cells to stimulate B cells. Increased ICAM-1 synthesis on B cells facilitates the cell to cell contact. Thus, the activation status of polyclonal B cells plays a critical role during the initiation of a specific antibody response.

The contribution of NK cell activity for the establishment of specific antibodies was, however, surprising. NK cells are part of the innate immune system and as such are involved in the first line of defense against pathogens. Most likely the cytokine pattern produced by NK cells upon activation is closely related to the initiation of a specific immune response. Thus, in one aspect the invention relates to a method of identifying an adjuvant by detecting NK cell activation. The NK cell activation assay may be carried out as described in the Examples below or using other known NK cell activity assays. It

is preferred, however than a mixed cell population such as PBMAC be used because of the likelihood that NK cell activation is an indirect effect. The assay is preferably useful for identifying immunostimulatory nucleic acids which are useful as adjuvants in human and other non-rat animals.

Cytokine induction was also identified as an important predictor of *in vivo* adjuvant activity. As there is a 2 log higher endotoxin sensitivity of human than mouse primary monocytes, some caution, however, is required to avoid endotoxin contamination of immunostimulatory nucleic acids used for testing in the human system (Hartmann G., and Kriegl A. M. 1999, *Gene Therapy* 6:893). Since TNF- α , IL-6 and IL-12 are produced by human monocytes in response to even low amounts of endotoxin, their value for high throughput *in vitro* screening assays is limited. On the other hand, human B cells and NK cells show only minor activation by endotoxin and thus are far more useful in testing for immunostimulatory activity.

Stimulation of cellular function in either NK or B cells (i.e., lytic activity, proliferation) requires a stronger immunostimulatory nucleic acid than the induction of activation markers at their surface (CD69, CD86). For both cell types, the use of cell surface activation markers showed a higher nonspecific background attributable to the phosphorothioate backbone compared to the functional assays. This high sensitivity of surface markers requires the use of low immunostimulatory nucleic acid concentrations for optimal discrimination between immunostimulatory nucleic acid of cellular activity. Thus, the use of surface markers allows the comparison of immunostimulatory nucleic acids with weak activity, while functional assays are preferred for comparing immunostimulatory nucleic acids with high activity. It is of note that the optimal immunostimulatory nucleic acid concentrations for stimulating B cells and NK cells differ. While 0.6 μ g/ml ODN is already maximal to stimulate B cells, optimal NK cell activation may require 6 μ g/ml ODN. Both B cell activation and NK cell functional activity were measured within freshly isolated PBMAC. It was previously found that highly purified human primary B cells are activated by CpG DNA. The existence of a direct effect of CpG DNA on NK cells is less clear, and a secondary mechanism mediated by another cell type within PBMAC might contribute to CpG-induced functional activity of NK cells.

The nucleic acids of the invention may be administered to a subject with an anti-microbial agent. An anti-microbial agent, as used herein, refers to a naturally-occurring or synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the invention will depend upon the type of microorganism with which the subject is infected or at risk of becoming infected. Anti-microbial agents include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as

"anti-infective agent", "anti-bacterial agent", "anti-viral agent", "anti-fungal agent", "anti-parasitic agent" and "parasiticide" have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Antibiotics are low molecular weight molecules which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more bacterial functions or structures which are specific for the microorganism and which are not present in host cells. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasitic agents kill or inhibit parasites.

Examples of anti-parasitic agents, also referred to as parasitocides useful for human administration include but are not limited to albendazole, amphotericin B, benzimidazole, bilisanol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, difloxamide fumarate, eflorentin, furozolidonum, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate, mefloquine, metronidazole, metronidazole, nifurtimox, oxamandiquin, paromomycin, pentamidine isethionate, piperazine, praziquantel, primaquine phosphate, proguanil, pyramel pneumonia, pyrimethamine-sulfonamides, pyrimethamine-sulfadiazole, quinine HCl, quinine sulfate, quinidine gluconate, spinosyn, sibogluconate sodium (sodium antimony gluconate), suramin, tetrahydropyrim, doxycycline, thiabendazole, tinidazole, trimethoprim-sulfamethoxazole, and pyrazinamide some of which are used alone or in combination with others.

[illegible][illegible]

Non-nucleic acid adjuvants include, for instance, adjuvants that create a depot effect, immune stimulating adjuvants, and adjuvants that create a depot effect and stimulate the immune system.

An adjuvant that creates a depot effect as used herein is an adjuvant that causes the antigen to be slowly released in the body, thus prolonging the exposure of immune cells to the antigen. This class of adjuvants includes but is not limited to alum (e.g., aluminum hydroxide, aluminum phosphate), or emulsion-based formulations including mineral oil, non-aqueous oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720, AltrAqualide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, CA; and PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC, Pharmaceuticals Corporation, San Diego, CA).

An immune stimulating adjuvant is an adjuvant that causes activation of a cell of the immune system. It may, for instance, cause an immune cell to produce and secrete cytokines. This class of adjuvants includes but is not limited to saponins purified from the bark of the *Q. sapotaria* tree, such as QS21 (a glycolipid that elicits in the 21st peak with HPLC fractionation; Aquila Biopharmaceuticals, Inc., Worcester, MA); poly[di(carboxylatophenyl)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, MT), muramyl dipeptide (MDP; Ribi) and threonyl-carrumyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmanin elongation factor (a purified *Leishmania* protein; Corixa Corporation, Seattle, WA).

Adjuvants that create a depot effect and stimulate the immune system are those compounds which have both of the above-identified functions. This class of adjuvants includes but is not limited to ISCOMs (immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia); SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21; SmithKline Beecham Biologicals [SBB], Rixensart, Belgium); SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium); non-ionic block copolymers that form micelles

such as CRL 1005 (these contain a linear chain of hydrophobic polyoxypropylene flanked by chains of polyoxyethylene; Vaxcel, Inc., Norcross, GA); and Syntex Adjuvant Formulation (SAP, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, CO).

The immunostimulatory nucleic acids are also useful as mucosal adjuvants. It has previously been discovered that both systemic and mucosal immunity are induced by mucosal delivery of CpG nucleic acids. The systemic immunity induced in response to CpG nucleic acids included both humoral and cell-mediated responses to specific antigens that were not capable of inducing systemic immunity when administered alone to the mucosa. Furthermore, both CpG nucleic acids and cholera toxin (CT, a mucosal adjuvant that induces a Th2-like response) induced CTL. This was surprising since with systemic immunization, the presence of Th2-like antibodies is normally associated with a lack of CTL (Schirmbeck *et al.*, 1995). Based on the results presented herein it is expected that the immunostimulatory nucleic acids will function in a similar manner.

Additionally, the immunostimulatory nucleic acids induce a mucosal response at both local (e.g., lung) and remote (e.g., lower digestive tract) mucosal sites. Significant levels of IgA antibodies are induced at distant mucosal sites by the immunostimulatory nucleic acids. CT is generally considered to be a highly effective mucosal adjuvant. As has been previously reported (Snider 1995), CT induces predominantly IgG1 isotype of antibodies, which are indicative of Th2-type response. In contrast, the

immunostimulatory nucleic acids are more Th1 with predominantly IgG2a antibodies, especially after boost or when the two adjuvants are combined. Th1-type antibodies in general have better neutralizing capabilities, and furthermore, a Th2 response in the lung is highly undesirable because it is associated with asthma (Kay, 1996; Hogg, 1997).

Thus the use of immunostimulatory nucleic acids as a mucosal adjuvant has benefits that other mucosal adjuvants cannot achieve. The immunostimulatory nucleic acids of the invention also are useful as mucosal adjuvants for induction of both a systemic and a mucosal immune response.

Mucosal adjuvants referred to as non-nucleic acid mucosal adjuvants may also be administered with the immunostimulatory nucleic acids. A non-nucleic acid mucosal adjuvant as used herein is an adjuvant other than a immunostimulatory nucleic acid that is capable of inducing a mucosal immune response in a subject when administered to a

mucosal surface in conjunction with an antigen. Mucosal adjuvants include but are not limited to Bacterial toxins e.g., Cholera toxin (CT), CT derivatives including but not limited to CT B subunit (CTB) (Wu et al., 1998; Tachibana et al., 1998); CTB53 (Val to Arg) (Fontana et al., 1995); CTB37 (Val to Lys) (Fontana et al., 1995); CTB104 (Tyr to Lys) (Fontana et al., 1995); CTB53/K63 (Val to Arg, Ser to Lys) (Fontana et al., 1995); CTB54 (Arg to His) (Fontana et al., 1995); CTB112K (Gln to Lys) (Fontana et al., 1995); CTB114 (Ser to Gln) (Fontana et al., 1995); CTB112K (Gln to Lys) (Fontana et al., 1995); CTB61P (Ser to Phe) (Fontana et al., 1997a, 1997b); CTB106 (Pro to Lys) (Douce et al., 1997; Fontana et al., 1995); and CTB63 (Ser to Lys) (Douce et al., 1997; Fontana et al., 1995). *Zonula occludens* toxin, zot, *Bacteroides* coli heat-labile enterotoxin, Labile Toxin (LT), LT derivatives including but not limited to LT B subunit (LTB) (Verweij et al., 1998); LTTK (Arg to Lys) (Komase et al., 1998; Douce et al., 1995); LTT6P (Ser to Phe) (Komase et al., 1998); LTT112K (Gln to Lys) (Komase et al., 1998); LTT118E (Gly to Gln) (Komase et al., 1998); LTT146B (Arg to Gln) (Komase et al., 1998); LTT192G (Arg to Gly) (Komase et al., 1998); LTTK63 (Ser to Lys) (Marchetti et al., 1998; Douce et al., 1997, 1999; Di Tommaso et al., 1996); and LTTK72 (Ala to Arg) (Giuliani et al., 1998). Pertussis toxin, PT. (Lysala et al., 1992; Spangler BD, 1992; Freytag and Clemmensen, 1999; Roberts et al., 1995; Wilson et al., 1995) including PT-9K/129G (Roberts et al., 1995; Cropley et al., 1995); Toxin derivatives (see below) (Holmgren et al., 1993; Verweij et al., 1998; Rappunli et al., 1995; Freytag and Clemmensen, 1999); Lipid A derivatives (e.g., monophosphoryl lipid A, MPL) (Sasaki et al., 1998; Vamout et al., 1998; Muramyl Dipeptide (MDP) derivatives (Fukushima et al., 1996; Ogawa et al., 1989; Michalek et al., 1983; Morisaki et al., 1983); Bacterial outer membrane proteins (e.g., outer surface protein A (OspA) lipoprotein of *Borrelia burgdorferi*, outer membrane proteins of *Neisseria meningitidis*) (Martino et al., 1999; Van de Vlegel et al., 1996); Oil-in-water emulsions (e.g., MF59) (Baruch et al., 1999; Verachor et al., 1999; O'Hagan, 1998); Aluminum salts (Saka et al., 1998, 1999); and Saponins (e.g., QS21) Aquila Biopharmaceuticals, Inc., Worcester, MA) (Sasaki et al., 1998; MacNeil et al., 1998); ISCOMS, MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, CA); the Sargis ISA series of Monomelic adjuvants (e.g., Montanide ISA 720; Aft Aquide, Paris, France); PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a nucleic-

forming agent; IDBC Pharmaceuticals Corporation, San Diego, CA); Syntex Adjuvant Formulation (SAF; Syntex Chemicals, Inc., Boulder, CO); poly(dicyclopentadienyl)phosphazene (PCPP polymer; Virus Research Institute, USA) and Leishmanin elongation factor (Corixa Corporation, Seattle, WA).

Immune responses can also be induced or augmented by the co-administration or co-linear expression of cytokines (Quelley & Mulligan, 1996; Chow et al., 1997; Griebler et al., 1997; Iwasaki et al., 1997; Kim et al., 1997) or B-7 co-stimulatory molecules (Iwasaki et al., 1997; Tsuji et al., 1997) with the immunostimulatory nucleic acids. The cytokines can be administered directly with immunostimulatory nucleic acids or may be administered in the form of a nucleic acid vector that encodes the cytokine, such that the cytokine can be expressed *in vivo*. In one embodiment, the cytokine is administered in the form of a plasmid expression vector. The term cytokine is used as a generic name for a diverse group of soluble proteins and peptides which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Examples of cytokines include, but are not limited to IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-18, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interferon- γ (γ -IFN), IFN- α , tumor necrosis factor (TNF), TGF- β , FLT-3 ligand, and CD40 ligand.

Cytokines play a role in directing the T cell response. Helper (CD4+) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including other T cells. Most mature CD4+ T helper cells express one of two cytokine profiles: Th1 or Th2. The Th1 subset promotes delayed-type hypersensitivity, cell-mediated immunity, and immunoglobulin class switching to IgG_{2b}. The Th2 subset induces humoral immunity by activating B cells, promoting antibody production, and inducing class switching to IgG₁ and IgE. In some embodiments, it is preferred that the cytokine be a Th1 cytokine.

The nucleic acids are also useful for redirecting an immune response from a Th2 immune response to a Th1 immune response. Redirection of an immune response from a Th2 to a Th1 immune response can be assessed by measuring the levels of cytokines produced in response to the nucleic acid (e.g., by inducing monocyte cells and other

cells to produce Th1 cytokines, including IL-12, IFN- γ and GM-CSF). The redirection or rebalance of the immune response from a Th2 to a Th1 response is particularly useful for the treatment or prevention of asthma. For instance, an effective amount for treating asthma can be that amount useful for redirecting a Th2 type of immune response that is associated with asthma to a Th1 type of response. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN- γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. The immunostimulatory nucleic acids of the invention cause an increase in Th1 cytokines which helps to rebalance the immune system, preventing or reducing the adverse effects associated with a predominantly Th2 immune response.

The nucleic acids are also useful for improving survival, differentiation, activation and maturation of dendritic cells. The immunostimulatory nucleic acids have the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells. Dendritic precursor cells isolated from blood by immunomagnetic cell sorting develop morphologic and functional characteristics of dendritic cells during a two day incubation with GM-CSF. Without GM-CSF these cells undergo apoptosis. The immunostimulatory nucleic acids are superior to GM-CSF in promoting survival and differentiation of dendritic cells (MHC II expression, cell size, granularity). The immunostimulatory nucleic acids also induce maturation of dendritic cells. Since dendritic cells form the link between the innate and the acquired immune system, by presenting antigens as well as through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local environment, the ability to activate dendritic cells with immunostimulatory nucleic acids supports the use of these immunostimulatory nucleic acid based strategies for *in vivo* and *ex-vivo* immunotherapy against disorders such as cancer and allergic or infectious diseases. The immunostimulatory nucleic acids are also useful for activating and inducing maturation of dendritic cells.

Immunostimulatory nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). ADCC can be performed using a immunostimulatory nucleic acid in combination with an antibody specific for a cellular

target, such as a cancer cell. When the immunostimulatory nucleic acid is administered to a subject in conjunction with the antibody the subject's immune system is induced to kill the tumor cell. The antibodies useful in the ADCC procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available. Examples of these antibodies are listed below among the list of cancer immunotherapies.

The immunostimulatory nucleic acids may also be administered in conjunction with an anti-cancer therapy. Anti-cancer therapies include cancer medications, radiation and surgical procedures. As used herein, a "cancer medication" refers to a agent which is administered to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medication is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medications for the treatment of cancer are described herein. For the purpose of this specification, cancer medications are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

As used herein, a "cancer medication" refers to an agent which is administered to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medication is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medications for the treatment of cancer are described herein. For the purpose of this specification, cancer medications are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers. Additionally, the methods of the invention are intended to embrace the use of more than one cancer medication along with the immunostimulatory nucleic acids. As an example, where appropriate, the immunostimulatory nucleic acids may be administered with a both a chemotherapeutic agent and an immunotherapeutic agent. Alternatively, the cancer medication may embrace an immunotherapeutic agent and a cancer vaccine, or a

- 101 -

chemotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine all administered to one subject for the purpose of treating a subject having a cancer or at risk of developing a cancer.

Cancer medications function in a variety of ways. Some cancer medications work by targeting physiological mechanisms that are specific to tumor cells. Examples include the targeting of specific genes and their gene products (i.e., proteins primarily) which are mutated in cancers. Such genes include but are not limited to oncogenes (e.g., Ras, Her2, bcl-2), tumor suppressor genes (e.g., EGFR, p53, Rb), and cell cycle targets (e.g., CDK4, p21, telomerase). Cancer medications can alternatively target signal transduction pathways and molecular mechanisms which are altered in cancer cells. Targeting of cancer cells via the epitopes expressed on their cell surface is accomplished through the use of monoclonal antibodies. This latter type of cancer medication is generally referred to herein as immunotherapy.

Other cancer medications target cells other than cancer cells. For example, some medications prime the immune system to attack tumor cells (i.e., cancer vaccines). Still other medications, called angiogenesis inhibitors, function by attacking the blood supply of solid tumors. Since the most malignant cancers are able to metastasize (i.e., exit the primary tumor site and seed a distal tissue, thereby forming a secondary tumor), medications that impede this metastasis are also useful in the treatment of cancer. Angiogenic mediators include basic FGF, VEGF, angiotensin, angiotensin, endostatin, TNF α , TNF-470, thrombospondin-1, platelet factor 4, CAI, and certain members of the integrin family of proteins. One category of this type of medication is a metalloproteinase inhibitor, which inhibits the enzymes used by the cancer cells to exit the primary tumor site and extravasate into another tissue.

Some cancer cells are antigenic and thus can be targeted by the immune system. In one aspect, the combined administration of immunostimulatory nucleic acids and cancer medications, particularly those which are classified as cancer immunotherapies, is useful for stimulating a specific immune response against a cancer antigen. A "cancer antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens, such as those present in cancer vaccines or those used to

- 102 -

prepare cancer immunotherapies, can be prepared from crude cancer cell extracts, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, or by partially purifying the antigens, using recombinant technology, or de novo synthesis of known antigens. Cancer antigens can be used in the form of immunogenic portions of a particular antigen or in some instances a whole cell or a tumor mass can be used as the antigen. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

The theory of immune surveillance is that a prime function of the immune system is to detect and eliminate neoplastic cells before a tumor forms. A basic principle of this theory is that cancer cells are antigenically different from normal cells and thus elicit immune reactions that are similar to those that cause rejection of immunologically incompatible allografts. Studies have confirmed that tumor cells differ, either qualitatively or quantitatively, in their expression of antigens. For example, "tumor-specific antigens" are antigens that are specifically associated with tumor cells but not normal cells. Examples of tumor specific antigens are viral antigens in tumors induced by DNA or RNA viruses. "Tumor-associated" antigens are present in both tumor cells and normal cells but are present in a different quantity or a different form in tumor cells. Examples of such antigens are oncofetal antigens (e.g., carcinoembryonic antigen), differentiation antigens (e.g., T and Tr antigens), and oncogene products (e.g., HER/neu).

Different types of cells that can kill tumor targets *in vitro* and *in vivo* have been identified: natural killer cells (NK cells), cytolytic T lymphocytes (CTLs), lymphokine-activated killer cells (LAKs), and activated macrophages. NK cells can kill tumor cells without having been previously sensitized to specific antigens, and the activity does not require the presence of class I antigens encoded by the major histocompatibility complex (MHC) on target cells. NK cells are thought to participate in the control of nascent tumors and in the control of metastatic growth. In contrast to NK cells, CTLs can kill tumor cells only after they have been sensitized to tumor antigens and when the target antigen is expressed on the tumor cells that also express MHC class I. CTLs are thought to be effector cells in the rejection of transplanted tumors and of tumors caused by DNA viruses. LAK cells are a subset of null lymphocytes distinct from the NK and CTL populations. Activated macrophages can kill tumor cells in a manner that is not antigen

dependent nor MHC restricted once activated. Activated macrophages are through to decrease the growth rate of the tumors they infiltrate. *In vitro* assays have identified other immune mechanisms such as antibody-dependent, cell-mediated cytotoxic reactions and lysis by antibody plus complement. However, these immune effector mechanisms are thought to be less important *in vivo* than the function of NK, CTLA, LAK, and macrophages *in vivo* (for review see Pissens, W.P., and David, J., "Tumor Immunology", in: *Scientific American Medicine*, Vol. 2, Scientific American Books, N.Y., pp. 1-13, 1996).

The goal of immunotherapy is to augment a patient's immune response to an established tumor. One method of immunotherapy includes the use of adjuvants. Adjuvant substances derived from microorganisms, such as bacillus Calmette-Guérin, heighten the immune response and enhance resistance to tumors in animals.

Immunotherapeutic agents are medicaments which derive from antibodies or antibody fragments which specifically bind or recognize a cancer antigen. As used herein a cancer antigen is broadly defined as an antigen expressed by a cancer cell. Preferably, the antigen is expressed at the cell surface of the cancer cell. Even more preferably, the antigen is one which is not expressed by normal cells, or at least not expressed to the same level as in cancer cells. Antibody-based immunotherapies may function by binding to the cell surface of a cancer cell and thereby stimulate the

endogenous immune system to attack the cancer cell. Another way in which antibody-based therapy functions is as a delivery system for the specific targeting of toxic

substances to cancer cells. Antibodies are usually conjugated to toxins such as ricin (e.g., from castor beans), colicistatinin and maytansinoids, to radioactive isotopes such as Iodine-131 and Yttrium-90, to chemotherapeutic agents (as described herein), or to biological response modifiers. In this way, the toxic substances can be concentrated in the region of the cancer and non-specific toxicity to normal cells can be minimized. In addition to the use of antibodies which are specific for cancer antigens, antibodies which bind to vasculature, such as those which bind to endothelial cells, are also useful in this invention. This is because generally solid tumors are dependent upon newly formed blood vessels to survive, and thus most tumors are capable of recruiting and stimulating the growth of new blood vessels. As a result, one strategy of many cancer medicaments

is to attack the blood vessels feeding a tumor and/or the connective tissues (or stroma) supporting such blood vessels.

The use of immunostimulatory nucleic acids in conjunction with immunotherapeutic agents such as monoclonal antibodies is able to increase long-term survival through a number of mechanisms including significant enhancement of ADCC (as discussed above), activation of natural killer (NK) cells and an increase in IFN γ levels. The nucleic acids when used in combination with monoclonal antibodies serve to reduce the dose of the antibody required to achieve a biological result.

Examples of cancer immunotherapies which are currently being used or which are in development are listed in Table C.

Table C

Cancer Immunotherapies in Development or on the Market		
MARKETER	BRAND NAME (GENERIC NAME)	INDICATION
IDEC/Genentech, Inc./Hoffmann-La Roche (first monoclonal antibody licensed for the treatment of cancer in the U.S.)	Rituxan [®] (rituximab, Mabthera) (IDEC-C2B8, chimeric murine/human anti-CD20 MAb)	non-Hodgkin's lymphoma
Genentech/Hoffmann-La Roche Cytogen Corp.	Herceptin, anti-H227MAB	breast cancer
Centocor/Ciba/Junonote	Quadrant (CYP-405) radiotherapeutic agent antibody	B-cell neoplasias
Centocor/Junonote	Prostate [®] (P-1A) (murine monoclonal antibody)	Adjuvant therapy for colorectal (Duke-C)
IDEC	Prostate [®] (P-1A) (chimeric murine monoclonal antibody)	Pancreatic, lung, breast, ovary
IntClone Systems	IDEC-Y2B4 (murine, anti-CD30) MAB labeled with Yttrium-90	non-Hodgkin's lymphoma
IntClone Systems	BB-93 (anti-Idioglyc MAB, mimics the CD-3 epitope) (with B10)	small cell lung
Techniclone International/Alpha Therapeutics	C225 (chimeric monoclonal antibody to epidermal growth factor receptor (EGFR))	Metastatic
Protein Design Labs	SMART M193 AB, humanized	non-Hodgkin's lymphoma
Techniclone Corporation/Cambridge Antibody Technology	anti-VEGF (Quincy [®])	Acute myeloid leukemia
Amgen Pharmaceuticals, Inc.	ATRA (AT-101)	non-Hodgkin's lymphoma
IntClone Systems	C2B5 (chimeric anti-CD19 monoclonal antibody) + cisplatin or radiation	Acute promyelocytic leukemia
		Head & neck, non-small cell lung cancer

Abnova, Canada	Ovexin (B3.15, anti-Hic-type CA125, mouse MAb)	Ovarian
Conley Pharma (clinical results have been positive, but the drug has been associated with significant bone marrow toxicity)	Bevac (anti-CD30 MAb labeled with ⁹⁰ Y)	non-Hodgkin's lymphoma
Abnova Pharmaceuticals, Inc.	ATRA-906	Kaposi's sarcoma
IDEC Pharmaceuticals Corp./Genentech	Rituxan [®] (MAb against CD20) plus B-Ab in combo. with chemotherapy	B cell lymphoma
Leukodiolin Oncology	DP-401 (mAb to the leukocyte antigen CD44v6)	Granulocytic leukemia (CLL)
Center of Molecular Immunology	1e16 (anti CD4, murine MAb) CTL	Cancer
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Breast, ovarian
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Prostate, non-small cell lung, pancreatic, breast
Medarex	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Acute myelogenous leukemia (AML)
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Renal and colon
Medarex	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Bx who have marrow purging in acute myelogenous leukemia (AML)
Medarex	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Acute myeloid leukemia
Medarex	MDX-22 (humanized bispecific antibody, MAb-conjugates) (complement cascade activator)	Acute myeloid leukemia
Cyrogen	OV103 (Vincristine-90 labeled antibody)	Ovarian
Cyrogen	OV103 (Vincristine-90 labeled antibody)	Prostate
Abnova Pharmaceuticals, Inc.	ATRA-906	non-Hodgkin's lymphoma
Glaxo Wellcome plc	33219p1 MAb that binds to EDG-40 (T _H 1A) presentation antigen on dendritic cells	non-small cell lung, prostate (adjunct)
Genentech	Anti-VEGF, VEGF-MABs (anti-VEGF antibodies)	Lung, breast, prostate, colorectal
Protein Design Labs	Zenopex (BMAAT Anti-Tie (T _H 2 receptor) Ab, humanized)	Leukemia, lymphoma
Protein Design Labs	BMAAT M193 Ab, humanized	Acute promyelocytic leukemia
ImClone Systems	C225 (chimeric anti-EGF monoclonal antibody) + bevac	Breast
ImClone Systems (licensed from BPI)	C225 (chimeric anti-EGF monoclonal antibody) + docetaxel	Prostate
ImClone Systems	C225 (chimeric anti-EGF monoclonal antibody) + adriamycin	Prostate
ImClone Systems	B3C2 (anti-Hic-type MAb, similar to Gb ₂ epitope)	Melanoma
Medarex	MDX-210 (humanized anti-HER-2 bispecific antibody)	Cancer

Medarex	MDX-220 (bispecific for tumor that expresses TAC-7)	Lung, colon, prostate, ovarian, endometrial, pancreatic and gastric
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Prostate
Medarex/Merck KGaA	MDX-441 (humanized anti-EGF receptor bispecific antibody)	EGF receptor cancer (head & neck, prostate, lung, bladder, cervical, ovarian)
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Comb. Therapy with G-CSF for various cancers, esp. breast
IDEC	ME18A01NE-2 (murine monoclonal antibody therapeutic vaccine)	Melanoma
IDEC	ME18A01NE-1 (murine monoclonal antibody therapeutic vaccine)	Melanoma
Immunomedics, Inc.	CEACIDE [®] (T-131)	Colorectal and other lymphoma
Novartis	Protargel [®] radioactive antibodies	non-Hodgkin's B cell lymphoma
Novopharm Biotech, Inc.	NovoMAB-02 (gonadotropin specific Ab)	Cancer
Techniclon Corporation/ Cambridge Antibody Technology	TNF (chimeric MAb to human antigen)	Brain
Techniclon International/ Cambridge Antibody Technology	TNF (chimeric MAb to human antigen)	Brain
Novopharm Biotech, Inc.	NovoMAB-02 (gonadotropin specific Ab)	Cancer
Genentech/Amgen/AMP	QIN-230 MAb	Colorectal
Merck KGaA	BM02-72000 (chimeric-EGF antigen)	Cancer
Immunomedics	LymphoCide (humanized IL2 antibody)	non-Hodgkin's B-cell lymphoma
Immunotec/AMP	CMA 676 (monoclonal antibody conjugate)	Acute myelogenous leukemia
Novopharm Biotech, Inc.	Memoparin-C	Colon, lung, pancreatic
Novopharm Biotech, Inc.	4B3 anti-Hic-type Ab	Melanoma, small-cell lung
Center of Molecular Immunology	1e16 (anti B3.15 humanized Ab)	Keloid immunotherapy
Center of Molecular Immunology	1e16 (anti B3.15 humanized Ab) for radiation immunotherapy	Colorectal
Genentech Biologics/Calera	BAB3 (chimeric antibody binding site)	Breast cancer
ImClone Systems/Chugai	PLK-2 (monoclonal antibody to final liver kinase-2 (PLK-2))	Tumor-associated angiogenesis
Immunotec, Inc.	Humanized MAb anti-drug conjugates	Small-cell lung
Medarex, Inc.	MDX-260 bispecific, targets CD-3	Melanoma, glioma

Procyon Biopharma, Inc.	ANA Ab	neuroblastoma
Protein Design Lab	SMART ID19 Ab	Cancer
Protein Design Lab/Novartis	SMART ABC Y84 Ab	B-cell lymphoma
Immunomedics, Inc.	ImmunAIP-CEA	Breast, lung, colon
		Colorectal

Yet other types of chemotherapeutic agents which can be used according to the

invention include Aminoglutethimide, Asparaginase, Buulfan, Carboplatin, Chlorambucil, Cytarabine HCl, Docetaxin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Flouxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna, Mitomycin (o,p'-DDD), Mitoxantrone HCl, Oestradiol, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Vincristine (n-AMSA), Azacitidine, Ertropoietin, Ifosfamide/Inmelamine (HMM), Interferon 2, Mitoguanazone (methyl-GAG; methyl glyoxal bis-guanylhydrazones; MGBG), Penicillin 2, Mitoguanazone (methyl-GAG; Semustine (methyl-CCNU), Teoposide (VM-26) and Vinorelbine sulfate.

Cancer vaccines are medicaments which are intended to stimulate an endogenous immune response against cancer cells. Currently produced vaccines predominantly activate the humoral immune system (i.e., the antibody dependent immune response). Other vaccines currently in development are focused on activating the cell-mediated immune system including cytotoxic T lymphocytes which are capable of killing tumor cells. Cancer vaccines generally enhance the presentation of cancer antigens to both antigen presenting cells (e.g., macrophages and dendritic cells) and/or to other immune cells such as T cells, B cells, and NK cells.

Although cancer vaccines may take one of several forms, as discussed infra, their purpose is to deliver cancer antigens and/or cancer associated antigens to antigen presenting cells (APC) in order to facilitate the endogenous processing of such antigens by APC and the ultimate presentation of antigen presentation on the cell surface in the context of MHC class I molecules. One form of cancer vaccine is a whole cell vaccine which is a preparation of cancer cells which have been removed from a subject, treated *ex vivo* and then reintroduced as whole cells in the subject. Lysates of tumor cells can

also be used as cancer vaccines to elicit an immune response. Another form cancer vaccine is a peptide vaccine which uses cancer-specific or cancer-associated small proteins to activate T cells. Cancer-associated proteins are proteins which are not exclusively expressed by cancer cells (i.e., other normal cells may still express these antigens). However, the expression of cancer-associated antigens is generally

consistently upregulated with cancers of a particular type. Yet another form of cancer vaccine is a dendritic cell vaccine which includes whole dendritic cells which have been exposed to a cancer antigen or a cancer-associated antigen *in vitro*. Lysates or membrane fractions of dendritic cells may also be used as cancer vaccines. Dendritic cell vaccines are able to activate antigen-presenting cells directly. Other cancer vaccines include ganglioside vaccines, heat-shock protein vaccines, viral and bacterial vaccines, and nucleic acid vaccines.

The use of immunostimulatory nucleic acids in conjunction with cancer vaccines provides an improved antigen-specific humoral and cell mediated immune response, in addition to activating NK cells and endogenous dendritic cells, and increasing IFN α levels. This enhancement allows a vaccine with a reduced antigen dose to be used to achieve the same beneficial effect. In some instances, cancer vaccines may be used along with adjuvants, such as those described above.

As used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses.

Other vaccines take the form of dendritic cells which have been exposed to cancer antigens *in vitro*, have processed the antigens and are able to express the cancer

antigens at their cell surface in the context of MHC molecules for effective antigen presentation to other immune system cells.

The immunostimulatory molecule acids are used in one aspect of the invention in conjunction with cancer vaccines which are dendritic cell based. A dendritic cell is a professional antigen presenting cell. Dendritic cells form the link between the innate and the acquired immune system by presenting antigens and through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local environment. Dendritic cells efficiently internalize, process, and present soluble specific antigen to which it is exposed. The process of internalizing and presenting antigen causes rapid upregulation of the expression of major histocompatibility complex (MHC) and costimulatory molecules, the production of cytokines, and migration toward lymphatic organs where they are believed to be involved in the activation of T cells.

Table D lists a variety of cancer vaccines which are either currently being used or are in development.

Table D

MANUFACTURER	Cancer Vaccine in Development or on the Market	BRAND NAME (GENERIC NAME)	INDICATION
Center of Molecular Immunology		BCP	Cancer
Center of Molecular Immunology			Ungliadite cancer vaccine
Center of Molecular Immunology		Anti-Hitogly	Cancer vaccine
ImCana Systems/Memorial Sloan-Kettering Cancer Center		Gp75 antigen	Melanoma
ImCana Systems/Memorial Sloan-Kettering Cancer Center		Anti-Hitogly Ab	Cancer vaccine
Progenics Pharmaceuticals, Inc.		GSK melanoma vaccine	Melanoma
Progenics Pharmaceuticals, Inc.		MDV granulocyte colony-stimulating factor vaccine	Lymphoma, colorectal, lung
Certa		H22100	Breast, ovarian
Abbott		Dysent	Ovarian
AVAX Technologies Inc.		H-Vac, autologous whole cell	Melanoma
AVAX Technologies Inc.		O-Vac, autologous whole cell	Ovarian
AVAX Technologies Inc.		L-Vac, autologous whole cell	Lymphoma-AML
Biontra Inc./Chiron		Therapep, FTH-KLH	Breast, Colorectal

Biontra Inc.	BLP23, MUC-1 peptide vaccine encapsulated in liposomal delivery system	Lung
Biontra Inc.	BLP23, MUC-1 peptide vaccine encapsulated in liposomal delivery system + Liposomal IL-2	Lung
Biontra Inc.	Liposomal Hitogly vaccine	Lymphoma B-cell malignancies
Bio Immunodex	Melanoma, cell lysate	Melanoma
Certa	Peptide antigens, microsphere delivery system and LAMP adjuvant	Breast
Certa	Peptide antigens, microsphere delivery system and LAMP adjuvant	Prostate
Certa	Peptide antigens, microsphere delivery system and LAMP adjuvant	Ovarian
Certa	Peptide antigens, microsphere delivery system and LAMP adjuvant	Lymphoma
Certa	Peptide antigens, microsphere delivery system and LAMP adjuvant	Lung
Virus Research Institute	Toxin/antigen recombinant delivery system	All cancers
Aplon Inc.	Gastric-TCR	T-cell lymphoma
Bayer/Nordic Research Institute A/S	MVA-based (vaccine virus) vaccine	Melanoma
BioCana Pharma/BioCana Vaccine	PACIS, BCG vaccine	Bladder
Camba Pharmaceuticals	TA-HPV	Cervical
Camba Pharmaceuticals	TA-CN	Cervical
Camba Pharmaceuticals	DISC-Virus, immunotherapy	Cancer
Pastor Merieux Connaught	ImmuCytat/TherCytat - BCG Immunotherapeutic (Bacillus Calmette-Guérin/Connaught), for intravesical treatment of superficial bladder cancer	Bladder

As used herein, chemotherapeutic agents enhance all other forms of cancer medications which do not fall into the categories of immunotherapeutic agents or cancer vaccines. Chemotherapeutic agents as used herein encompass both chemical and biological agents. These agents function to inhibit a cellular activity which the cancer cell is dependent upon for continued survival. Categories of chemotherapeutic agents include alkylating/alkaloid agents, antineoplastic, hormones or hormone analogs, and miscellaneous antineoplastic drugs. Most if not all of these agents are directly toxic to cancer cells and do not require immune stimulation. Combination chemotherapy and

Johnson & Johnson	Lezantha	Chemithas	Cancer, Breast Cancer, Early Cell, Leukemia
Verax	Paceas	Pacilizer	Kidney Sarcoma
Sequus	Doxil	Doxetidine, Liposomal	Kid Cancer
Sequus	Cosyza	Doxetidine, Liposomal	Kid Cancer
Schering AG	Finidra	Platinabion	Leukemia
Pharmacia & Upjohn	Pharmabion	Epirubicin	Lung/Breast Cancer
Chiron	Dipocyt	Dipocyt	Neoplastic Meningitis
Zeneca	ZD 1033	ZD 1839	Non Small Cell Lung Cancer, Renal Cancer
BASF	LJ 79533	Bis-Naphthalimide	Oncology
BASF	LJ 103793	Dibenzos	Ovarian/Breast Cancer
Shering Plough	Cosyza	Diaminodiol, Liposome	Pancreatic Cancer, Non Small Cell Lung Cancer
Libby	Gancor	Ganciclovir	Breast, Bladder and Ovarian
Zeneca	ZD 0479/Assumed	ZD 0479/Assumed	Platinum based NSCL, ovarian etc.
Vancomycin	VM 115	VM 115	Prostatic Cancer
Nycomed	Secord, 125 Rapid 86	Lofline Sodal	Prostatic Cancer
Ameslab			
Apurum	Cdk2/cdk2 Inhibitors	cdk2/cdk2 Inhibitors	Solid Tumors
Apurum	PARP Inhibitors	PARP Inhibitors	Solid Tumors
Chironacis	D4189	Doxiflaminide	Solid Tumors
British Myers	UPF (1-segular/linear)	UPF (1-segular/linear)	Solid Tumors
Seib			
Sandoz	Krasin	Krasin	Solid Tumors
Aas Medical	182r/Assumed	182r/Assumed	Solid Tumors
British Myers	182r/Assumed	182r/Assumed	Solid Tumors
Seib			
British Myers	Venon	Teniposide	Solid Tumors
Seib			
British Myers	Pargapala	Carboplatin	Solid Tumors
Seib			
British Myers	Pargapala	Carboplatin, Squalid	Solid Tumors
Seib			
British Myers	Pargapala	Carboplatin	Solid Tumors
Seib			
British Myers	Vopexide	Bosopexide	Solid Tumors/Melanoma
Seib			
Zeneca	ZD 9331	ZD 9331	Solid Tumors, Advanced Colorectal
Chugai	Taxotere	Doxetidine	Solid Tumors, Breast Cancer
Roche Pharmacia	Taxotere	Doxetidine	Solid Tumors, Breast Cancer
Gilead Wellcome	Proliferation of granules	Proliferation of endothelial	T Cell Leukemia/T lymphoma
British Myers	Taxotere	Taxotere	T Cell Leukemia
Seib	Taxotere	Taxotere	Taxot Follow up

In one embodiment, the methods of the invention use immunostimulatory nucleic acids as a replacement to the use of IFN α therapy in the treatment of cancer. Currently, some treatment protocols call for the use of IFN α . Since IFN α is produced following the

administration of some immunostimulatory nucleic acids, these nucleic acids can be used to generate IFN α endogenously.

The invention also includes a method for inducing antigen non-specific innate immune activation and broad spectrum resistance to infectious challenge using the immunostimulatory nucleic acids. The term antigen non-specific innate immune activation as used herein refers to the activation of immune cells other than B cells and for instance can include the activation of NK cells, T cells or other immune cells that can respond in an antigen independent fashion or some combination of these cells. A broad spectrum resistance to infectious challenge is induced because the immune cells are in active form and are primed to respond to any invading compound or microorganism. The cells do not have to be specifically primed against a particular antigen. This is particularly useful in biodefense, and the other circumstances described above such as travellers.

The stimulation index of a particular immunostimulatory nucleic acid can be tested in various immune cell assays. Preferably, the stimulation index of the immunostimulatory nucleic acid with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^3H uridine in a murine B cell culture, which has been contacted with 20 μM of nucleic acid for 20h at 37°C and has been pulsed with 1 μCi of ^3H uridine; and harvested and counted 4h later as described in detail in PCT Published Patent Applications PCT/US95/01570 (WO 96/02555) and PCT/US97/19791 (WO 98/18810) claiming priority to U.S. Serial Nos. 08/386,063 and 08/890,774, filed on February 7, 1995 and October 30, 1997 respectively. For use *in vivo*, for example, it is important that the immunostimulatory nucleic acids be capable of effectively inducing an immune response, such as, for example, antibody production.

Immunostimulatory nucleic acids are effective in non-rodent vertebrates.

Different immunostimulatory nucleic acid can cause optimal immune stimulation depending on the type of subject and the sequence of the immunostimulatory nucleic acid. Many vertebrates have been found according to the invention to be responsive to the same class of immunostimulatory nucleic acids, sometimes referred to as human specific immunostimulatory nucleic acids. Rodents, however, respond to different nucleic acids. As shown herein an immunostimulatory nucleic acid causing optimal

stimulation in humans may not generally cause optimal stimulation in a mouse and vice versa. An immunostimulatory nucleic acid causing optimal stimulation in humans often does, however, cause optimal stimulation in other animals such as cow, horses, sheep, etc. One of skill in the art can identify the optimal nucleic acid sequences useful for a particular species of interest using routine assays described herein and/or known in the art, using the guidance supplied herein.

The immunostimulatory nucleic acids may be directly administered to the subject or may be administered in conjunction with a nucleic acid delivery complex. A nucleic acid delivery complex shall mean a nucleic acid molecule associated with (e.g. initially or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g., B cell surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.

Delivery vehicles or delivery devices for delivering antigen and nucleic acids to surfaces have been described. The immunostimulatory nucleic acid and/or the antigen and/or other therapeutics may be administered alone (e.g., in saline or buffer) or using any delivery vehicles known in the art. For instance the following delivery vehicles have been described: Cochleates (Gould-Fogerite et al., 1994, 1996); Emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Morris et al., 1993, Carlson et al., 1991, Hu et al., 1998, Morein et al., 1999); Liposomes (Childers et al., 1999, Michalek et al., 1989, 1992, de Haan 1995a, 1995b); Live bacterial vectors (e.g., *Salmonella*, *Escherichia coli*, *Bacillus edwards-guerini*, *Shigella*, *Lactobacillus*) (Hesse et al., 1996, Pourwela et al., 1998, Chutfield et al., 1993, Stover et al., 1991, Nugent et al., 1998); Live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex) (Gallichan et al., 1993, 1995, Moss et al., 1996, Nugent et al., 1998, Flenner et al., 1988, Morrow et al., 1999); Microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); Nucleic acid vaccines (Fyfe et al., 1993, Kulkarni et al.,

1997, Sasaki et al., 1998, Okada et al., 1997, Ishii et al., 1997); Polymers (e.g. carboxymethylcellulose, chitosan) (Hamaeima et al., 1998, Jabbel-Gill et al., 1998); Polymer rings (Wyatt et al., 1998); Protosomes (Vancott et al., 1998, Lowell et al., 1998, 1996, 1997); Sodium Fluoride (Hsieh et al., 1998); Transgenic plants (Tedder et al., 1998, Mason et al., 1998, Haq et al., 1995); Virosomes (Gluck et al., 1992, Mangardi et al., 1995, Cysz et al., 1998); Virus-like particles (Jiang et al., 1999, Leibl et al., 1998). Other delivery vehicles are known in the art and some additional examples are provided below in the discussion of vectors.

The term effective amount of a immunostimulatory nucleic acid refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a immunostimulatory nucleic acid for inducing mucosal immunity is that amount necessary to cause the development of IgA in response to an antigen upon exposure to the antigen, whereas that amount required for inducing systemic immunity is that amount necessary to cause the development of IgG in response to an antigen upon exposure to the antigen. Combined with the teachings provided herein, by choosing among the various active compounds and weighting factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular immunostimulatory nucleic acid being administered, the antigen, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular immunostimulatory nucleic acid and/or antigen and/or other therapeutic agent without necessitating undue experimentation.

Subject doses of the compounds described herein for mucosal or local delivery typically range from about 0.1 µg to 10 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween. More typically mucosal or local doses range from about 10 µg to 5 mg per administration, and most typically from about 100 µg to 1 mg, with 2 - 4 administrations being spaced days or weeks apart. More typically, immune stimulant

- 117 -

doses range from 1 µg to 10 mg per administration, and most typically 10µg to 1 mg, with daily or weekly administrations. Subject doses of the compounds described herein for parenteral delivery for the purpose of inducing an antigen-specific immune response, wherein the compounds are delivered with an antigen but not another therapeutic agent are typically 5 to 10,000 times higher than the effective mucosal dose for vaccine adjuvant or immune stimulant applications, and more typically 10 to 1,000 times higher, and most typically 20 to 100 times higher. Doses of the compounds described herein for parenteral delivery for the purpose of inducing an innate immune response or for increasing ADCC or for inducing an antigen specific immune response when the immunostimulatory nucleic acids are administered in combination with other therapeutic agents or in specialized delivery vehicles typically range from about 0.1 µg to 10 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween. More typically parenteral doses for these purposes range from about 10 µg to 5 mg per administration, and most typically from about 100 µg to 1 mg, with 2 - 4 administrations being spaced days or weeks apart. In some embodiments, however, parenteral doses for these purposes may be used in a range of 5 to 10,000 times higher than the typical doses described above.

For any compound described herein the therapeutically effective amount can be initially determined from animal models. A therapeutically effective dose can also be determined from human data for CpG oligonucleotides which have been tested in humans (human clinical trials have been initiated) and for compounds which are known to exhibit similar pharmacological activities, such as other mucosal adjuvants, e.g., LT and other adjuvants for vaccination purposes, for the mucosal or local administration. Higher doses are required for parenteral administration. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

- 118 -

For use in therapy, an effective amount of the immunostimulatory nucleic acid can be administered to a subject by any mode that delivers the nucleic acid to the desired surface, e.g., mucosal, systemic. Administering the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferred routes of administration include but are not limited to oral, parenteral, intranasal, intramuscular, intramedullary, inhalation, ocular, vaginal, and rectal.

For oral administration, the compounds (i.e., immunostimulatory nucleic acids, antigens and other therapeutic agents) can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable excipients, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginate acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as

talco or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions.

Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymers or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, emulsified, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

The immunostimulatory nucleic acids and optionally other therapeutics and/or antigens may be administered *per se* (nude) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric,

- 121 -

phosphoric, maleic, acetic, salicylic, p-toluenesulphonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzoic sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Storable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.5-2% w/v). Storable preservatives include benzalkonium chloride (0.003-0.03% w/v), chlorobutanol (0.3-0.9% w/v), parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

10 The pharmaceutical compositions of the invention contain an effective amount of an immunostimulatory nucleic acid and optionally antigens and/or other therapeutic agents optionally included in a pharmaceutically-acceptable carrier. The term

pharmaceutically-acceptable carrier means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term carrier denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being concomitantly with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutic efficiency.

20 The immunostimulatory nucleic acids useful in the invention may be delivered in mixtures with additional adjuvant(s), other therapeutics, or antigen(s). A mixture may consist of several adjuvants in addition to the immunostimulatory nucleic acid or several antigen or other therapeutics.

25 A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular adjuvants or antigen selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

- 122 -

5 The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, nature and severity of the disorder, age and body weight of the patient, different doses may be necessary. The administration of a given dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units. Multiple administration of doses at specific intervals of weeks or months apart is usual for boosting the antigen-specific response.

15 Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactic-glycolic), copolyoxalates, poly(vinylacetate), polyesteramides, polyorthoesters, poly(hydroxybutyric acid), and polyurethides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di- and tri-glycerides; hydrogel release systems; elastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) ocular systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Examples

Materials and Methods

Oligodeoxynucleotides: Native phosphodiester and phosphorothioate-modified ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon (Specialty Products (Miffland, MA). ODN were tested for endotoxin using the LAL-assay (LAL-assay BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml). For *in vitro* assays, ODN were diluted in TE-buffer (10 mM Tris, pH 7.0, 1 mM EDTA), and stored at -20° C. For *in vivo* use, ODN were diluted in phosphate buffered saline (0.1 M PBS, pH 7.3) and stored at 4°C. All dilutions were carried out using pyrogen-free reagents.

Isolation of human PBMC and cell culture: Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (Histopaque-1077; Sigma Chemical Co., St. Louis, MO) as described (Hartmann et al., 1999 Proc. Natl. Acad. Sci USA 96:9305-10). Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, NY) (complete medium). Cells (final concentration 1×10^6 cells/ml) were cultured in complete medium in a 5% CO₂ humidified incubator at 37°C. ODN and LPS (from *Salmonella typhimurium*, Sigma Chemical Co., St. Louis, MO) or anti-IgM were used as stimuli. For measurement of human NK lytic activity, PBMC were incubated at 5×10^6 /well in 24-well plates. Cultures were harvested after 24 hours, and cells were used as effectors in a standard 4 hours ⁵¹Cr-release assay against K562 target cells as previously described (Ballas et al., 1996 J. Immunol. 157:1840-1845). For B cell proliferation, 1 µCi of ³H thymidine was added 18 hours before harvest, and the amount of ³H thymidine incorporation was determined by scintillation counting at day 5. Standard deviations of the triplicate wells were < 5%.

Flow cytometry on human PBMC: Surface antigens on primary PBMC were stained as previously described (Hartmann et al., 1998 J. Pharmacol. Exp. Ther. 285:920-928). Monoclonal antibodies to CD3 (UCHL1), CD14 (M5E2), CD19 (B43), CD56 (B159), CD69 (FN50) and CD86 (2331 [FUN-1]) were purchased from Pharmingen, San Diego, CA. IgG1,κ (MOFC-21) and IgG2a,κ (Hartmann et al., 1999 Proc. Natl. Acad. Sci USA 96:9305-10) were used to control for non-specific staining. NK cells were identified by CD56 expression on CD3, CD14 and CD19 negative cells, whereas B cells were identified by expression of CD19. Flow cytometric data of 10000 cells per sample were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA). The viability of cells within the FSC/SSC gate used for analysis was examined by propidium iodide staining (2 µg/ml) and found to be higher than 98%. Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stanford, CA).

Results:

Example 1: CpG-dependent stimulation of human B cells depends on methylation and ODN length

Human PBMC were obtained from normal donors and cultured for five days at 2×10^6 cells/well with the indicated concentrations of the indicated ODN sequences. As shown in Table F, human PBMCs proliferate above the background when cultured with a variety of different CpG ODN, but also show some proliferation even with ODN that do not contain any CpG motifs. The importance of unmethylated CpG motifs in providing optimal immune stimulation with these ODN is demonstrated by the fact that ODN 1840 (SEQ ID NO. 83) induces 56,603 counts of ³H-thymidine incorporation whereas the same T-rich ODN with the CpG motifs methylated (non-CpG), 1979 (SEQ ID NO. 222), induces lower, but still increased over background, activity (only 18,618 counts) at the same concentration of 0.6 µg/ml. The reduced proliferation at higher ODN concentrations may be an artifact of the cells becoming exhausted under these experimental conditions or could reflect some toxicity of the higher ODN concentrations. Interestingly, shorter ODN containing CpG motifs, such as the 13-14mers 2015 and 2016, are less stimulatory despite the fact that their molar concentration would actually be higher since the ODNs were added on the basis of mass rather than molarity. This demonstrates that ODN length may also be an important determinant in the immune

- 125 -

effects of the ODN. A non-CpG ODN but slight T-rich ODN (about 30% T), 1982 (SEQ ID NO. 225), caused only a small amount of background cell proliferation.

Table F

Oligo Concentration			
ODN#	0.15 µg/ml	0.6 µg/ml	2 µg/ml
Cues only	648	837	799
1840 (SEQ ID NO. 83)	5744	56,603	31,787
2016 (SEQ ID NO. 256)	768	4607	20,497
1979 (SEQ ID NO. 222)	971	18,618	29,246
1892 (SEQ ID NO. 135)	787	10,078	22,850
2010 (SEQ ID NO. 250)	849	20,741	8,054
2012 (SEQ ID NO. 252)	2586	62,955	52,462
2013 (SEQ ID NO. 253)	1043	47,960	47,231
2014 (SEQ ID NO. 254)	2700	50,708	46,625
2015 (SEQ ID NO. 255)	1059	23,239	36,119

Numbers represent cpm of ³H-thymidine incorporation for cultures of human PBMCs set up as described above.

Example 2. Concentration-dependent activation of human NK cell activity with thymidine-rich ODN.

- 126 -

Human PBMCs were cultured for 24 hours with a panel of different CpG or non-CpG ODN at two different concentrations, and then tested for their ability to kill NK target cells as described previously (Galles et al., 1996 *J. Immunol.* 157:1840-1845). Killing is measured as lytic units, or L.U. The human donor used in this experiment had a background level of 3.69 L.U. which increased to 180.36 L.U. using the positive control, IL-2. A CpG oligo, 2006 (SEQ ID NO. 246), induced high levels of NK lytic function at a low concentration of 0.6, and a lower level at a concentration of 6.0. Surprisingly, a T-rich ODN in which the CpG motifs of 2006 were methylated (ODN at 2117 (SEQ ID NO. 358)) or inverted to GpCs (ODN 2137 (SEQ ID NO. 886)) retained strong immune stimulatory function at the higher ODN concentrations, as shown in Table G. These concentration-dependent immune stimulatory effects are not a general property of the phosphorothioate backbone since the experiments described below demonstrate that a poly-A ODN, is nonstimulatory above background levels. Some stimulation is seen with a 24-base long ODN in which all of the base positions are randomized so that A, C, G, and T will occur at a frequency of 25% in each of the base positions (ODN 2182 (SEQ ID NO. 432)). However, the stimulatory effect of such a 24-base ODN is greatly enhanced if it is pure poly-T, in which case stimulation is also seen at the lowest concentration of 0.6 µg/ml (ODN 2183 (SEQ ID NO. 433)). In fact, the stimulatory activity of ODN SEQ ID NO. 433 at this low concentration is higher than that of any other ODN tested at this low concentration, aside from the optimal human immune stimulatory ODN of SEQ ID NO. 246. In fact, the higher concentration of ODN SEQ ID NO. 433 stimulated more NK activity than any other phosphorothioate ODN except for the strong CpG ODN 2142 (SEQ ID NO. 890), which was marginally higher. If the G content of ODN SEQ ID NO. 246 is increased relative to the T content by addition of more Gs, thus resulting in a decrease in the proportion of T nucleotides the immune stimulatory effect of the ODN is reduced (see ODN 2132 (SEQ ID NO. 373)). Thus, the T content of an ODN is an important determinant of its immune stimulatory effect. Although a poly-T ODN is the most stimulatory of the non-CpG ODN, other bases are also important in determining the immune stimulatory effect of a non-CpG ODN. ODN 2131 (SEQ ID NO. 372), in which slightly more than half of the bases are T and in which there are no Gs, is immune stimulatory at a concentration of 6 µg/ml but has less activity than other T-rich ODN. If the 6 A's in ODN 2131 (SEQ ID NO. 372)

are replaced by 6 Gs, the immune stimulatory effect of the ODN can be increased (see ODN 2130 (SEQ ID NO. 371)).

Table G

HUMAN PBL CULTURED OVERNIGHT WITH OLIGOS

[illegible]

- 129 -

+ 2131 (6.0 ug/ml)	2.29	6.73	7.30	18.02	32.73	49.06	5.08
+ 2136 (0.3 ug/ml)	2.50	5.26	8.20	15.95	26.64	33.07	2.31
+ 2136 (1.0 ug/ml)	5.91	10.99	17.31	26.97	50.64	63.78	10.84
+ 2137 (0.3 ug/ml)	2.36	4.00	6.65	12.94	24.13	38.86	2.58
+ 2137 (1.0 ug/ml)	3.72	9.55	17.15	34.55	52.27	65.33	11.58
+ 2158 (0.3 ug/ml)	1.25	2.36	6.90	16.39	15.63	29.82	1.17
+ 2158 (1.0 ug/ml)	4.73	7.26	11.07	15.55	30.80	43.71	4.16
+ 2118 (0.6 ug/ml)	1.55	3.38	6.85	13.36	20.15	27.71	1.13
+ 2118 (6.0 ug/ml)	2.65	3.88	9.29	12.19	22.47	28.99	1.34

Example 3: Induction of B cell proliferation by T-rich non-CpG ODN.

To assess the ability of T-rich ODN to activate B cell proliferation, human PBMCs were stained with the cytoplasmic dye CFSE, incubated with five days with the indicated ODN at either 0.15 or 0.3 ug/ml, and then analyzed by flow cytometry. B cells were identified by gating on cells positive for the lineage marker CD19. CpG ODN 2006 was a strong inducer of B cell proliferation, and this effect was reduced if the CpG motifs were methylated or inverted to GpC as shown in Figure 1 at an ODN concentration of 0.3 ug/ml. The base composition of the ODN appears to be important in determining the immune stimulatory effect. Reducing the T content of an ODN substantially reduces immune stimulatory effect, as exemplified by ODN 2177 (SEQ ID NO. 427) in which 6 of the Ts present in ODN 2137 (SEQ ID NO. 886) have been switched to A's, resulting in a greatly reduced immune stimulatory effect. The importance of T's in the immune stimulatory effect of an ODN is also shown by comparison of ODN 2116 (SEQ ID NO. 357) and 2181 (SEQ ID NO. 431), which differ in the 3' end of the ODN. ODN 2181, in which the 3' end is poly-T is more stimulatory

- 130 -

than ODN 2116, in which the 3' end is poly-C, despite the fact that both ODN have a TCGTCG at the 5' end.

Example 4: B Cell Proliferation Induced by TG Oligonucleotides

The stimulatory effects of TG motifs are shown in Figure 2. ODN 2137 has the identical base composition as ODN 2006, but the CG motifs have all been inverted to GC's resulting in a CG-free nucleic acid. ODN does however contain 6 TG dinucleotides. In ODN 2177, all the TG dinucleotides of ODN 2137 have been changed to AG. Although ODN 2177 contains only 6 adenines, it is virtually nonstimulatory at a concentration of 0.2 ug/ml. For comparison, an ODN 24 bases in length in which each position is randomized to be any of the four bases (ODN 2182) induces > 12% of B cells to proliferate at a concentration of 0.2 ug/ml. These results indicate that the stimulatory effects of ODN 2137 are not simply those of a phosphorothioate backbone, but relate to the presence of TG dinucleotides.

In order to determine the effect of varying the number of TG dinucleotide motifs, ODN 2200 and ODN 2202 were compared, as shown in Figure 2. Both ODN contain 18 Ts and 6 Gs, but in ODN 2200 all of the Gs are consecutive, so that there is only one TG dinucleotide, whereas in ODN 2202, the Gs are split up as CG dinucleotides throughout the ODN so that there are three TGs. ODN 2202 is significantly more stimulatory than ODN 2200, consistent with the model that at least three TG motifs in an ODN are required for optimal stimulatory activity. It is likely that even higher levels of stimulation could be achieved if the TG motifs had been optimized as taught herein.

Example 5: Effects of TTG versus TTG motif.

Figure 3 shows the results of experiments conducted to study TG content in terms of the relative levels of Ts versus Gs as it relates to the stimulatory effect of an ODN. The Figure shows that an ODN in which all of the bases are randomized to be either T or G (ODN 2188 (SEQ ID NO. 905)) is nonstimulatory at a concentration of 0.2 ug/ml, similar to an ODN in which all of the bases are randomized to be either A or G (ODN 2189 (SEQ ID NO. 906)). However, at the higher concentration of 2 ug/ml, the randomized TTG ODN 2188 is significantly more stimulatory. This latter level of stimulation is still lower than that which occurs with a totally randomized ODN (ODN 2182 (SEQ ID NO. 432)). The highest stimulation at low concentrations is seen with an

CpG ODN 2006 (SEQ ID NO.: 246). PBMC (2×10^6 cells/ml) of a blood donor (r=2) were incubated with the indicated concentrations of ODNs 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 5126 (SEQ ID NO.: 1058), and 5162 (SEQ ID NO.: 1094). Cells were incubated for 48h at 37°C as described above and stained with mAb for CD19 (B cell marker) and CD86 (B cell activation marker, B7-2). Expression was measured by flow cytometry.

Using different concentrations of ODNs, we showed (Fig. 4) that T-rich ODNs without a CpG motif can induce stimulation of human B cells. ODN 5126 (SEQ ID NO.: 1058) which contains only a single poly-T sequence but is greater than 50% T, caused high levels of human B cell activation. Although there are some similarities to SEQ ID NO.: 246 (e.g. more than 80% T/G content), this ODN clearly lacks any known immunostimulatory CpG motif. Surprisingly, for all tested T-rich ODNs, the highest stimulatory index was obtained at concentrations between 3 and 10 μ g/ml. The highest stimulatory index of the tested ODNs was achieved by CpG/T-rich ODN SEQ ID NO.: 246 at 0.4 μ g/ml. Interestingly, the activity decreased at high concentrations.

Poly A, Poly C and Poly T sequences were synthesized and tested for biological activity. PBMC (2×10^6 cells/ml) of one representative donor (r=3) were stimulated as described above by 0.4 μ g/ml, 1.0 μ g/ml or 10.0 μ g/ml of the following ODNs: 2006 (SEQ ID NO.: 246), 2196 (SEQ ID NO.: 913) (Poly T, 18 bases), 2194 (SEQ ID NO.: 911) (Poly T, 27 bases), 5162 (SEQ ID NO.: 1094) (Poly T, 30 bases), 5163 (SEQ ID NO.: 1093) (Poly A, 30 bases), 5168 (SEQ ID NO.: 1096) (Poly C, 30 bases) and 5169 (SEQ ID NO.: 1097) (Poly C, 30 bases). Expression of the activation marker CD86 (B7-2) on CD19-positive B cells was measured by flow cytometry.

Fig. 5 demonstrates that the length of the sequence, at least for Poly T ODNs, has an important impact on its activity. A Poly T sequence containing only 18 bases (SEQ ID NO.: 913) was shown to be less stimulatory than one with 27 bases (SEQ ID NO.: 911) or one with 30 bases (SEQ ID NO.: 1094) with a clear rank of stimulation: SEQ ID NO.: 1094 > SEQ ID NO.: 911 > SEQ ID NO.: 913. Poly A (SEQ ID NO.: 1095) or Poly C (SEQ ID NO.: 1097) sequences, in contrast, do not induce activation of human B cells. Surprisingly it was also discovered that Poly C sequences (SEQ ID NO.: 1096) can activate human B cells at least at high concentrations (10 μ g/ml) (Fig. 5).

Two other T-rich ODNs, namely 1982 (SEQ ID NO.: 225) and 2041 (SEQ ID NO.: 282) lacking CpG motifs were tested for their effect on human B cells. PBMC (r=2) were incubated with the indicated concentrations of ODN 2006 (SEQ ID NO.: 246), 1982 (SEQ ID NO.: 225) and 2041 (SEQ ID NO.: 282) as described above. B cell activation (expression of the activation marker CD86) was measured by flow cytometry.

Fig. 6 demonstrates that T-rich non-CpG ODN are immunostimulatory at concentrations higher than 1 μ g/ml. Incorporation of a CpG motif into 1982 enhanced the immunostimulatory activity. Elongation with a Poly T sequence did not enhance the immunostimulatory activity of this already T-rich ODN but rather, decreased the activation potential slightly.

Example 7: Immunostimulation of non-CpG ODNs is reflected in the enhancement of NK activation, NK cytotoxicity and monocyte activation

NK cells as well as monocytes were tested for their response to non-CpG ODNs. PBMC (2×10^6 cells/ml) were incubated with 6 μ g/ml of the following ODNs (r=4): 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058). After 24h of cultivation at 37°C cells were harvested and stained with mAb for CD3 (T cell marker), CD56 (NK cell marker) and CD69 (early activation marker) as described above. Expression of CD69 on CD56-positive NK cells was measured by flow cytometry.

Fig. 7 shows that for Poly T ODNs similar effects can be observed as described in Fig. 5. The stimulation of NK cells, like B cells, may be influenced by the length of the ODN. ODN 2183 (SEQ ID NO.: 433) (21 bases) induced activation of NK cells but to a lesser extent than the longer ODN 2194 (SEQ ID NO.: 911) (27 bases), as measured by enhanced expression of the early activation marker CD69. ODN 5126 (SEQ ID NO.: 1058) was also demonstrated to activate human NK cells (Fig. 7).

It is believed that the anti-tumor activity of CpG ODNs can be assessed by the ability of the ODN to enhance NK-mediated cytotoxicity *in vitro*. ODNs containing at the 5' and 3' ends stretches of Poly G were shown to result in the highest induction of cytotoxicity (Ballas, Z. K., et al. 1996 *J. Immunol.* 157:1840). To investigate the influence of non-CpG T-rich ODN on NK cytotoxicity, we analyzed the effect of the

ODNs 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058) on NK-mediated lysis (Fig. 8). NK-mediated lysis of K-562 target cells was measured after overnight incubation of PBMC with 6 μ g/ml of the ODN 2006 (SEQ ID NO.: 246), SEQ ID NO.: 911 (SEQ ID NO.: 911) (Poly T, 27 bases) and 5126 (SEQ ID NO.: 1058) as described above. SEQ ID NO.: 1058 demonstrated small increases in lysis by human NK cells as compared to no ODN. SEQ ID NO.: 911 and SEQ ID NO.: 246 enhanced human NK cell cytotoxicity to an even higher extent.

Previous reports demonstrated that not only NK cells but also NKT cells are mediators of cytotoxic responses to tumor cells (14). We, therefore, looked at the potential activation of human NKT cells by T-rich non-CpG ODN. PBMC of one representative donor (n=2) were incubated with 6 μ g/ml ODN 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 913) and 5126 (SEQ ID NO.: 1058) for 24h as described above. Activation of NKT cells was measured by flow cytometry after staining of cells with mAb for CD3 (T cell marker), CD56 (NKT cell marker) and CD69 (early activation marker). Shown is the expression of CD69 on CD3 and CD56 double-positive cells (NKT cells).

In Fig. 9, SEQ ID NO.: 911 as well as SEQ ID NO.: 1058 were found to stimulate NKT cells. Similar to NK cells SEQ ID NO.: 911 (Poly T) was more active than SEQ ID NO. 1058. In addition, as described above for B cells and NK cells, the length of the ODN has some influence on the immunostimulatory potential, with the longer ODN having stronger effects on NKT cells. Similar results were observed for human T cells.

Another type of cell of the immune system involved in fighting infectious is the monocytes. These cells release upon activation a variety of cytokines and can mature into dendritic cells (DC), professional antigen-presenting cells (Reitt, L., J. Brostoff, and D. Males, 1998, *Immunology*, Mosby, London). Fig. 10 shows activation of human monocytes after culturing of PBMC with different ODNs. PBMC (2x10⁶ cells/ml) were incubated with 6 μ g/ml 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2178 (SEQ ID NO.: 1096), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911), 5126 (SEQ ID NO.: 1058) and 5163 (SEQ ID NO.: 1095) overnight at 37°C as

described above (n=3). Cells were harvested and stained for CD14 (monocyte marker) and CD80 (B7-1, activation marker). Expression was measured by flow cytometry.

As demonstrated above for NK and B cells, T-rich sequences (e.g., SEQ ID NO.: 433, SEQ ID NO.: 911) of different length induce monocyte stimulation but have different levels of activity e.g., SEQ ID NO.: 433 > SEQ ID NO.: 911. Poly A (SEQ ID NO.: 1095) as well as Poly C (SEQ ID NO.: 1096 (2178) sequences, in contrast, did not lead to activation of monocytes (measured by the upregulation of CD80 at a concentration of 6 μ g/ml ODN).

Example 8: Induction of cytokine release by non-CpG ODNs

Next the ability of different T-rich ODNs to influence the cytokine milieu was examined. PBMC (3x10⁶ cells/ml) were cultured for 24h with or without 6 μ g/ml of the indicated ODNs or 1 μ g/ml LPS as positive control (n=2). After incubation supernatants were collected and TNF α measured by ELISA as described above and the results are shown in Fig. 11. PBMC were cultured with the indicated ODNs (1.0 μ g/ml) as described in Fig. 11 and IL-6 was measured in the supernatants by ELISA and the results are shown in Fig. 12.

Fig. 11 and 12 demonstrate that T-rich non-CpG and T-rich/CpG ODNs can induce the secretion of the pro-inflammatory cytokines TNF α and IL-6. For both cytokines, ODN 5126 (SEQ ID NO.: 1058) was found in most assays to be as potent as ODN 2194 (SEQ ID NO.: 911). It is known that CpG ODNs influence the Th1/Th2 balance by preferentially inducing Th1 cytokines (Krieg, A. M. 1999 *Biochemistry et Biophysics Acta* 932:1-7). To test whether T-rich ODN caused a similar shift to Th1 cytokines, IFN γ production in PBMC was measured. In a first set of experiments, it was demonstrated that, as described for IL-6 and TNF α , ODNs SEQ ID NO.: 1058 and SEQ ID NO.: 911 induced the release of comparable amounts of this Th1 cytokine IFN γ . In addition, it was demonstrated that another pro-inflammatory cytokine, IL-1 β , was released upon culture of PBMC with these two ODNs. Although the amount of these cytokines induced by the T-rich ODN lacking CpG motifs was less than when CpG ODN SEQ ID NO.: 246 was used the amounts induced by T-rich ODN were significantly higher than the control.

Example 2-11**Introduction:**

- An optimal CpG motif for immune system activation in non-rodent vertebrates is described herein. A phosphodiester oligonucleotide containing this motif was found to strongly stimulate CD86, CD40, CD54 and MHC II expression, IL-6 synthesis and proliferation of primary human B-cells. These effects required internalisation of the oligonucleotide and endosomal maturation. This CpG motif was associated with the sustained induction of the NF- κ B p50/p65 heterodimer and of the transcription factor complex activating protein-1 (AP-1). Transcription factor activation by CpG DNA was preceded by increased phosphorylation of the stress kinases c-jun NH κ terminal kinase (JNK) and p38, and of activating transcription factor-2 (ATF-2). In contrast to CpG, signaling through the B-cell receptor led to activation of extracellular receptor kinase (ERK) and to phosphorylation of a different isoform of JNK.

Materials and Methods:

- 15 **Oligodeoxynucleotides:** Unmodified (phosphodiester, PS) and modified nucleoside-resistant (phosphorothioate, PS) ODN were purchased from Operon Technologies (Alameda, CA) and Hydrion Specialty Products (Milford, MA). The sequences used are provided in Table II. B. cell DNA and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, MO. Genomic DNA samples were purified by extraction with phenol-chloroform-isoamyl alcohol (25/24/1) and ethanol precipitation. DNA was purified from endotoxin by repeated extraction with etion x-114 (Sigma Chemical Co., St. Louis, MO) and tested for endotoxin using the LAL-assay (LAL-assay BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml) and the high sensitivity assay for endotoxin described earlier (lower detection limit 0.0014 EU/ml) (Hartmann G., and Kriegl A. M. 1999. CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Therapy* 6:893). Endotoxin content of DNA samples was below 0.0014 U/ml. B. cell and calf thymus DNA were made single stranded before use by boiling for 10 minutes, followed by cooling on ice for 5 minutes. DNA samples were diluted in TE-buffer using pyrogen-free reagents.

30

Table II: Oligonucleotide panel used¹

	Name (SEQ ID NO)	Sequence 5' to 3'
Starting sequence	PE 2079 (330)	TCG ACG TTC CCC CCC CCC CC
Middle base	PE 2100 (341)	TCG GCG TTC CCC CCC CCC CC
	PE 2082 (323)	TCG CCG TTC CCC CCC CCC CC
Human CpG motif	PE 2080 (321)	TCG TCG TTC CCC CCC CCC CC
5' flanking base	PE 2105 (346)	GCG TCG TTC CCC CCC CCC CC
	PE 2107 (348)	ACG TCG TTC CCC CCC CCC CC
	PE 2104 (345)	CCG TCG TTC CCC CCC CCC CC
3' flanking base	PE 2098 (339)	TCG TCG CTC CCC CCC CCC CC
	PE 2099 (340)	TCG TCG GTC CCC CCC CCC CC
	PE 2083 (324)	TCG TCG ATC CCC CCC CCC CC
First CpG deleted	PE 2108 (349)	CTG TCG TTC CCC CCC CCC CC
Second CpG deleted	PE 2106 (347)	TCG TCA TTC CCC CCC CCC CC
Methylation	PE 2095 (336)	TZG TZG TTC CCC CCC CCC CC
	PE 2094 (335)	TCG TCG TTC CCC CCC ZCC CC
Non-CpG control of 2080	PE 2078 (319)	TGC TGC TTC CCC CCC CCC CC
	PE 2101 (342)	GGC CTT TTC CCC CCC CCC CC
PS form of 2080	PS 2116 (357)	TCG TCG TTC CCC CCC CCC CC
Additional CpG motifs	PE 2059 (300)	TCG TCG TTC TGT GGT TTT GTG GTT
Best PS	PS 2006 (246)	TCG TCG TTC TGT GGT TTT GTG GTT
Methylated 2006	PS 2117 (358)	TZG TZG TTC TGT ZGT TTT GTZ GTT

¹PE, phosphodiester; PS, phosphorothioate; bold, base exchange; bold Z, methylated cytidine; underlined, CpG dinucleotides.

Cell preparation and cell cultures: Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) as described (Hartmann G., et al 1996 *Antisense Nucleic Acid Drug Dev* 6:291). Cells were suspended in RPMI 1640 culture medium supplemented with 10 % (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, NY) (complete medium). All compounds were purchased endotoxin-tested. Viability was determined before and after incubation with ODN by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometric analysis). In all experiments, 96 % to 99 % of PBMC were viable. Cells (final concentration 1 x 10⁶ cells/ml) were cultured in complete medium in a 5 % CO₂ humidified incubator at 37°C. Different oligonucleotides (see table I, concentration as indicated in the figure legends), LPS (from salmonella typhimurium, Sigma Chemical Co., St. Louis, MO) or anti-IgM were used as stimuli. Chloroquine (5 µg/ml; Sigma Chemical Co., St. Louis, MO) was used to block endosomal maturation/acidification. At the indicated time points, cells were harvested for flow cytometry as described below.

For signal transduction studies, human primary B-cells were isolated by immunomagnetic cell sorting using the VARIOMACS technique (Miltenyi Biotec Inc., Auburn, CA) as described by the manufacturer. In brief, PBMC obtained from buffy coats of healthy blood donors (Elmer L. DeGowin Blood Center, University of Iowa) were incubated with a microbeads-conjugated antibody to CD19 and passed over a positive selection column. Purity of B-cells was higher than 95%. After stimulation, whole cellular extracts (Western blot) and nuclear extracts (EMSA) for signal transduction studies were prepared.

For CysG binding protein studies, Ramos cells (human Burkitt lymphoma B cell line, ATCC CRL-1923 or CRL-1596; Intervirology 5: 319-334, 1975) were grown in complete medium. Untreated cells were harvested and cytosolic protein extracts were prepared and analyzed for the presence of CysG oligonucleotide binding proteins by EMSA and UV-crosslink as described below.

Flow cytometry: Staining of surface antigens was performed as previously described (Hartmann G. et al. 1998 *J Pharmacol Exp Ther* 285:920). Monoclonal

antibodies to HLA-DR were purchased from Immunotech, Marseille, France. All other antibodies were purchased from Pharmingen, San Diego, CA: mAbs to CD19 (B43), CD40 (5C3), CD54 (HAS8), CD86 (2331 (FUN-1)), IgG₁ (MOFC-21) and IgG_{2b} were used to control for specific staining. Intracellular cytokine staining for IL-6 was performed as described (Hartmann G., and Krieg A. M. 1999. CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Therapy* 6:893). In brief, PBMC (final concentration 1 x 10⁶ cells/ml) were incubated in the presence of brefeldin A (final concentration 1 µg/ml, Sigma Chemical Co., St. Louis, MO). After incubation, cells were harvested and stained using a FITC-labeled mAb to CD19 (B43), a PE-labeled rat anti-human IL-6 mAb (MQ2-6A3, Pharmingen) and the Fix and Perm Kit (Caltag Laboratories, Burlingame, CA). Flow cytometric data of 5000 cells per sample were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Non-viable cells were excluded from analysis by propidium iodide staining (2 µg/ml). Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stamford, CA).

Proliferation assay: CFSE (5-(and-6-) carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, USA) is a fluorescein-derived intracellular fluorescent label which is divided equally between daughter cells upon cell division. Staining of cells with CFSE allows both quantification and immunophenotyping (phycoerythrin-labeled antibodies) of proliferating cells in a mixed cell suspension. Briefly, PBMC were washed twice in PBS, resuspended in PBS containing CFSE at a final concentration of 5 µM, and incubated at 37°C for 10 minutes. Cells were washed three times with PBS and incubated for five days as indicated in the figure legends. Proliferating CD19-positive B-cells were identified by decreased CFSE content using flow cytometry.

Preparation of whole cell, nuclear and cytosolic protein extracts: For Western blot analysis, whole cell extracts were prepared. Primary B-cells were treated with medium, the phosphodiester oligonucleotides 2080 (SEQ ID NO.: 321) or 2078 (SEQ ID NO.: 319) at 30 µg/ml, or anti-IgM (10 µg/ml). Cells were harvested, washed twice with ice-cold PBS containing 1 mM Na₂VO₄, resuspended in lysis buffer (150 mM NaCl, 10 mM TRIS pH 7.4, 1 % NP40, 1 mM Na₂VO₄, 50 mM NaF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml aprotinin, 5 mg/ml pepstatin, 50 µg/ml

- 141 -

phenylmethylsulfonylfluoride (PMSF), incubated for 15 min on ice and spun at 14000 rpm for 10 min. The supernatant was frozen at -80°C. For the preparation of nuclear extracts, primary B-cells were resuspended in hypotonic buffer (10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 0.05 % NP40, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml antipain, 5 mg/ml pepstatin). After 15 minutes incubation on ice, the suspension was centrifuged at 1000 x g for 5 minutes. The pelleted nuclei were resuspended in extraction buffer (20 mM HEPES (pH 7.9), 450 mM NaCl, 50 mM NaF, 20% glycerol, 1 mM EDTA, 1 mM BDTA, 1 mM DTT, 1 mM PMSF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml antipain, 5 mg/ml pepstatin) and incubated on ice for one hour. The nuclear suspension was centrifuged for 10 minutes at 16,000 g at 4°C. Supernatant was collected and stored at -80°C. Cytosolic extracts for the CpG binding protein studies were prepared from unstimulated Ramos cells, which were lysed with hypotonic buffer as described for the preparation of the nuclear extract. After centrifugation, the supernatant was removed as cytoplasmic fraction and stored at -80°C. Protein concentrations were measured using a Bradford protein assay (Bio-Rad, Hercules, CA) according to the manufacturer.

Western blot analysis: Equal concentrations of whole cell protein extracts (25 µg/lane) were boiled in SDS sample buffer (50 mM Tris-Cl, pH 6.8; 1% β -mercaptoethanol; 2% SDS; 0.1% bromophenolblue; 10% glycerol) for 4 min before being subjected to electrophoresis on a 10 % polyacrylamide gel containing 0.1 % SDS (SDS-PAGE). After electrophoresis, proteins were transferred to Immobilon-P transfer membranes (Millipore Corp, Bedford, MA). Blots were blocked with 5 % nonfat dry milk. Specific antibodies against the phosphorylated form of extracellular receptor kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 and activating transcription factor-2 (ATF-2) were used (New England Biolabs, Beverly, MA). Blots were developed in enhanced chemiluminescence reagent (ECL; Amersham International, Aylesbury, UK) according to the manufacturer's recommended procedure.

Electrophoretic mobility shift assay (EMSA): To detect the DNA-binding activity of the transcription factor activator protein-1 (AP-1) and NF- κ B, nuclear extracts (1 µg/lane) were analyzed by EMSA using the α ODN β 5' GAT CTA GTG AATG AGT CAG CCG GAT C 3' (SEQ ID NO.: 838) containing the AP-1 binding sequence, and the NF- κ B URE from the c-myc promoter region 5' TCG AAG AAG TCC GGG TTT TCC

- 142 -

CCA ACC CCC C 3' (SEQ ID NO.: 1142), as probes. ODNs were end labeled with T4 polynucleotide kinase (New England Biolabs) and (γ -³²P) ATP (Amersham, Arlington Heights, IL). Binding reactions were performed with 1 µg nuclear protein extract in DNA-binding buffer (10 mM Tris-HCl (pH 7.5), 40 mM MgCl₂, 20 mM EDTA, 1 mM dithiothreitol, 8% glycerol and 100 - 400 µg of poly (dI-dC) with 20,000 - 40,000 cpm labeled ODN in 10 µl total volume. Specificity of the NF- κ B bands was confirmed by competition studies with cold oligonucleotides from unrelated transcription factor binding sites (10 - 100 ng). For the supershift assay, 2 µg of specific antibodies for c-Rel, p50 and p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added into the reaction mixture for 30 min before the radiolabeled probe was added. Following incubation for 30 minutes at room temperature loading buffer was added and the probes were electrophoresed on a 6 % polyacrylamide gel in Tris-borate-EDTA running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0). Gels were dried and then autoradiographed.

UV-crosslinking and denaturing protein electrophoresis: Nuclear extracts were incubated with labeled phosphodiester oligonucleotide as described for the EMSA. DNA-protein complexes were crosslinked with UV-light in a Stratalinker (Stratagene) for 10 minutes. Probes were mixed with SDS-sample buffer, boiled for 10 minutes and loaded on a 7.5% SDS-PAGE. The gel was dried on Whatman paper and autoradiographed. Plotting the distance against the molecular weight of the marker proteins yielded a standard curve which was used to calculate the approximate molecular weight of the crosslinked protein-ODN complexes. The molecular weight of the oligonucleotide was subtracted from this value to give the size.

Example 9: Identification of an Optimal CpG motif for use alone or in combination with a T-rich ODN

Phosphorothioate oligonucleotides containing the murine CpG motif GACGTT (SEQ ID NO.: 1143) (for example 1826 (SEQ ID NO.: 69)) and used at concentrations which are active in murine B-cells (Yi A. K., Chang M., Pechham D. W., Krig A. M., and Ashman R. F. 1998. CpG oligodeoxynucleotides reduce mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J Immunol* 160:5898), have showed little or no immunostimulatory activity on human immune cells. At higher

concentrations this ODN was found to demonstrate some stimulatory effect on human B cells.

In earlier studies on B-cell activation in mice, it was found that a CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines and preferably the 6mer motif 5' GACGGT 3' (SEQ ID NO:1143) was optimal for a phosphodiester oligonucleotide to be active (Krieg A. M., et al. 1995 *Nature* 374:546, Yi A. K., Chang M., et al., 1998 *J Immunol* 160:4898).

In order to identify an optimal motif for stimulation of an immune response in humans and non-rodent vertebrates we designed a series of ODN and tested their activity. First we designed a 20 mer phosphodiester oligonucleotide with a TC dinucleotide at the 5' end preceding the optimal murine CpG motif 5' GACGGT 3' (SEQ ID NO: 1143) and followed by a poly C tail (2079: 5' TCG ACG TTC CCC CCC CCC 3' (SEQ ID NO.: 320)). This oligonucleotide if added to human primary B-cells under the same conditions as found to be optimal for E. coli DNA (repeated addition at 0 hours, 4 hours and 18 hours, 30 µg/ml for each time point) stimulated high levels of CD86 expression on human primary B-cells after two days. To determine the structure-function relationship of the CpG motifs, we replaced the bases adjacent to the CpG dinucleotides while maintaining the two CpG dinucleotides within the sequence.

Exchange of the adenine located between both CpG dinucleotides by thymidine (2080 (SEQ ID NO.: 321)) resulted in slightly higher activity. Replacement by guanosine (2100 (SEQ ID NO.: 341)) or cytidine (2082 (SEQ ID NO.: 323)) at this position showed no major changes compared to 2079 (SEQ ID NO.: 320). In contrast, replacement of the thymidine 3' to the second CpG dinucleotide by the purines guanosine (2099 (SEQ ID NO.: 340)) or adenine (2083 (SEQ ID NO.: 324)) resulted in a major drop in activity of the oligonucleotide, while the pyrimidine cytidine caused only a minor decrease. The thymidine immediately 5' to the first CpG dinucleotide was also important.

Replacement of the thymidine by any other base (2105 (SEQ ID NO.: 346), guanosine; 2107 (SEQ ID NO.: 348), adenine; 2104 (SEQ ID NO.: 345), cytidine) led to a marked decrease in activity of the oligonucleotide. Elimination of the first (2108 (SEQ ID NO.: 349)) or the second (2106 (SEQ ID NO.: 347)) CpG dinucleotide also partially reduced the activity.

The addition of more 5' GTCGTT 3' (SEQ ID NO.: 1144) CpG motifs to the phosphodiester oligonucleotide containing the 6mer duplex CpG motif 5' TCGTCGTT 3' (SEQ ID NO:1143), 2080 (SEQ ID NO.: 321)) did not further enhance CD86 expression on B-cells (2059 (SEQ ID NO.: 300)). An oligonucleotide with the same

sequence as 2080 (SEQ ID NO.: 321) but with a phosphorothioate backbone showed no activity above background (2116 (SEQ ID NO.: 357)). This was surprising since the phosphorothioate backbone has been reported to greatly stabilize oligonucleotides and enhance CpG-induced stimulation (Krieg A. M., Yi A. K., Matson S., Waldschmidt T. J., Bishop G. A., Teasdale R., Korenzky G. A., and Kliman D. M. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546). We therefore performed further structure-function analysis of phosphorothioate oligonucleotides containing the 5' GTCGTT 3' (SEQ ID NO:1144) and 5' TCGTCGTT 3' (SEQ ID NO:1145) motifs, which showed that additional CpG motifs (2006 (SEQ ID NO.: 246)) tended to increase the activity of phosphorothioate oligonucleotides.

Purified B-cells isolated from peripheral blood by immunomagnetic cell sorting were activated by CpG DNA to the same extent as unpurified B-cells within PBMC. Thus, activation of B-cells is a primary response and not a secondary effect caused by cytokines secreted by other cells.

In addition to the co-stimulatory molecule CD86, the functional stage of B-cells is characterized by other surface markers. For example, activated T helper cells stimulate B-cells by CD40 ligation, the intercellular adhesion molecule-1 (ICAM-1, CD54) mediates binding to other immune cells, and major histocompatibility complex II (MHC II) is responsible for antigen presentation. We found that B cell expression of CD40, CD54 and MHC II was upregulated by the CpG oligonucleotide 2080 (SEQ ID NO.: 321). The non-CpG control oligonucleotide 2078 (SEQ ID NO.: 319) showed no activity compared to medium alone.

When PBMC were incubated for 5 days in the presence of 2080 (SEQ ID NO.: 321) (added at 0 hours, 4 hours, 18 hours and every subsequent morning), it was intriguing that a subpopulation of lymphocytes increased in cell size (FSC) and became more granular (SSC). To examine if this subpopulation represented proliferating B-cells, we stained freshly isolated PBMC with CFSE (5-(and-6-) carboxyfluorescein diacetate succinimidyl ester) at day 0 and incubated them for 5 days with 2080 (SEQ ID NO.: 321)

as above. CFSE is a fluorescent molecule that binds irreversibly to cell proteins. Each cell division decreases CFSE stain by 50 %. Cells staining low with CFSE (proliferating cells) were found to be mainly CD19-positive B-cells. The oligonucleotide 2080 (SEQ ID NO.: 321) induced 60 to 70 % of CD19 positive B-cells to proliferate within 5 days.

5 The control oligonucleotide 2078 (SEQ ID NO.: 319) induced less than 5 % of B-cells to proliferate. Proliferating B-cells (CFSE low) showed a larger cell size (FSC) and higher granularity.

Proliferating B-cells expressed higher levels of CD86 than non-proliferating cells (not shown). In agreement with this finding, the oligonucleotide panel tested above for induction of CD86 expression resulted in an almost identical pattern of B-cell proliferation. Replacement of the 3' thymidine reduced activity more than changing the thymidine in the middle position.

Example 16: B-cell activation requires endosomal maturation/acidification

15 It has previously been shown that chloroquine, an inhibitor of endosomal acidification, blocks CpG-mediated stimulation of murine antigen presenting cells and B-cells, while not influencing LPS-mediated effects (Hacker H, et al 1998 *Embo J* 17:6230, Yi A. K. et al 1998 *J Immunol* 160:4735, Macchiarelli D, E., and Manzoni L. 1998 *J Immunol* 160:1122). We found that the addition of 5 µg/ml chloroquine completely blocked CpG DNA-mediated induction of CD86 expression on primary B-cells (MFI CD86: 2006 (SEQ ID NO.: 246), 4.7 vs 1.4; B. cell DNA, 3.4 vs. 1.4; medium only, 0.9; *ns*). Furthermore, chloroquine completely inhibited the induction of B-cell proliferation by the phosphorothioate oligonucleotide 2006 (SEQ ID NO.: 246) measured with the CFSE proliferation assay as well as with the standard. These results suggest that as with murine cells, activation of human B-cells by CpG DNA requires the uptake of DNA in endosomes and subsequent endosomal acidification.

Example 11: Analysis of sub-cellular events resulting upon human B cell stimulation with optimal human ODN.

30 Since the CpG motif requirement for maximal B-cell activation is substantially different between mouse (GACGTT) (SEQ ID NO:143) and humans (TCGTCGCTT) (SEQ ID NO:145), we were interested if the basic intracellular signaling events are

comparable. Rapid induction of NF- κ B binding activity has been found earlier in murine B-cells and macrophages (Shaoxy K. J., et al 1996 *J Immunol* 157:2116, Yi A. K. et al 1998 *J Immunol* 160:4735). To investigate the NF- κ B response to CpG DNA in humans, human primary B-cells were isolated from peripheral blood by immunomagnetic cell sorting and incubated with the CpG oligonucleotide 2080 (SEQ ID NO.: 321), the non-CpG control oligonucleotide 2078 (SEQ ID NO.: 319), or medium. At the indicated time points, cells were harvested and nuclear extracts were prepared. In the presence of CpG oligonucleotide, NF- κ B binding activity was increased within one hour and maintained up to 18 hours (latest time point examined). The non-CpG control oligonucleotide 2078 (SEQ ID NO.: 319) did not show enhanced NF- κ B activity compared to cells incubated with medium only. The NF- κ B band was identified by cold competition, and shown to consist of p50 and p65 subunits by supershift assay.

The activating protein-1 (AP-1) transcription factor is involved in the regulation of immediate early genes and cytokine expression (Karin M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 270:16483). In murine B-cells, AP-1 binding activity is induced in response to CpG DNA (Yi A. K., and Kiehl A. M. 1998. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J Immunol* 161:4493). To determine whether this transcription factor would also be induced by CpG DNA in humans, we examined AP-1 DNA binding activity in human primary B-cells. Cells were incubated with the CpG oligonucleotide 2080 (SEQ ID NO.: 321) or the control oligonucleotide 2078 (SEQ ID NO.: 319). Nuclear extracts were prepared and the AP-1 binding activity was analyzed by EMSA. AP-1 binding activity was enhanced within one hour, and increased up to 18 hours (latest time point examined), showing a sustained response.

25 Since AP-1 activity is induced by many stimuli (Angel P., and Karin M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1072:129), we were interested in signal transduction pathways upstream of AP-1. The AP-1 transcription factor complex integrates different mitogen activated protein kinase (MAPK) pathways (Karin M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 270:16483). Western blots were performed using whole cell extracts from primary B-cells incubated with the CpG oligonucleotide 2080 (SEQ ID NO.: 321), the control 2078 (SEQ ID NO.: 319), or

medium only. Specific antibodies to the phosphorylated form of JNK, p38, ATF-2 and ERK were used. Strong induction of JNK phosphorylation was found 30 min and 60 min after exposure to CpG-DNA, while the non-CpG oligonucleotide showed no activity above background. The protein kinase p38, another stress activated protein kinase (SAPK), was also phosphorylated in response to CpG DNA within 60 min. ATF-2, a substrate of both p38 and JNK (Gupta S., Campbell D., Derijard B., and Davis R. J. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267:389) and a component of the AP-1 complex, showed weak phosphorylation after 30 min which increased after 60 min. CpG DNA failed to induce substantial phosphorylation of ERK. In contrast, anti-IgM, stimulating the B-cell receptor, did trigger phosphorylation of ERK. Anti-IgM activated different isoforms of JNK than CpG DNA.

Example 12: Assay for *in vivo* adjuvant activity.

An *in vitro* screening assay to identify ODN useful as an adjuvant *in vivo* in humans and other non-rodent animals was developed. Since we saw not only quantitative but also qualitative differences in activities of different CpG ODN in mice, we first screened a panel of CpG and non-CpG control ODN on mouse cells to find *in vitro* assays with reliable and strong correlation to *in vivo* adjuvant activity with hepatitis B surface antigen (HBsAg). We then systematically tested a panel of more than 250 ODN in corresponding human assays to identify sequences with *in vitro* immunostimulatory activity. We next examined if the ODN with the highest activity in these human assays also activate B cell proliferation in chimpanzees and monkeys, and finally, if they are active as adjuvants with HBsAg in chimpanzees and cynomolgus monkeys *in vivo*. These studies revealed that the sequence, number and spacing of individual CpG motifs contribute to the immunostimulatory activity of a CpG phosphorothioate ODN. An ODN with a TC dinucleotide at the 5' end followed by three 6mer CpG motifs (5' GTCGTT 3') separated by TT dinucleotides consistently showed the highest activity for human, chimpanzees, and rhesus monkey leukocytes. Chimpanzees or monkeys vaccinated once against hepatitis B with this CpG ODN adjuvant developed 15 times higher anti-HBe antibody titers than those receiving vaccine alone.

Materials and Methods

Oligonucleotides: Phosphorothioate-modified ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). ODN were tested for endotoxin using the LAL-assay (LAL-assay BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml). For *in vitro* assays, ODN were diluted in TE-buffer (10 mM Tris, pH 7.0, 1 mM EDTA), and stored at -20° C. For *in vivo* use, ODN were diluted in phosphate buffered saline (0.1 M PBS, pH 7.3) and stored at 4° C. All dilutions were carried out using pyrogen-free reagents.

Mouse spleen cell cultures: Spleens were removed from 6-12 week old female BALB/c (The Jackson Laboratory), 2 x 10⁶ splenocytes were cultured with 0.2 µM ODN for 4 hours (TNF-α) or 24 hours (IL-6, IFN-γ, IL-12), and cytokines were detected by ELISA as previously described (Yi A. K., Klinman D. M., Martin T. L., Matsen S., and Krieg A. M. 1996. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol* 157:5394). To evaluate CpG-induced B cell proliferation, spleen cells were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lymphocyte M^c (Cedarlane Laboratories, Hornby, ON, Canada), cultured for 44 hours with the indicated ODN, and then pulsed for 4 hours with 1 µCi of ³H thymidine as described previously (Krieg A. M., Yi A. K., Matson S., Waldschmidt T. J., Bishop G. A., Teasdale R., Koretzky G. A., and Klinman D. M. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546). To examine NK cell lytic activity murine spleen cells were depleted of B cells using magnetic beads coated with goat anti-mouse Ig as previously detailed (Ballas Z. K., and Rasmussen W. 1993. Lymphokine-activated killer cells. VII. IL-4 induces an NK1.1⁺CD8^αβ⁺ TCR-αβ B220⁺ lymphokine-activated killer subset. *J Immunol* 150:17). Cells were cultured at 5 x 10⁶/well in 24-well plates and harvested at 18 hours for use as effector cells in a standard 4 hour ⁵¹Cr-release assay against YAC-1 target cells. One unit (LU) was defined as the number of cells needed to effect 30 % specific lysis.

Immunization of mice against HBsAg and evaluation of the humoral response: Groups of 6-8 week old female BALB/c mice (n = 5 or 10, Charles River, Montreal, QC) were immunized against HBsAg as previously described (Davis H. L., et

at 1998 *J Immunol* 160:870). In brief, each mouse received a single IM injection of 50 μ l PBS containing 1 μ g recombinant HBsAg (Medix Biotech, Foster City, CA) and 10 μ g of CpG ODN or non-CpG ODN as a sole adjuvant or combined with alum (Allydregel "85", Superfos Biosector, Vedbaek, Denmark; 25 mg Al³⁺/mg HBsAg). Control mice were immunized with HBsAg without adjuvant or with alum. Plasma was recovered from mice at various times after immunization and Abx specific to HBsAg (anti-HBx) were quantified by end-point dilution ELISA assay (in triplicate) as described previously (Davis H. L. et al 1998 *J Immunol* 160:870). End-point titer was defined as the highest plasma dilution that resulted in an absorbance value (OD450) two times higher than that of non-immune plasma with a cut-off value of 0.05.

Isolation of primate PBMC and cell culture: Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers, chimpanzees or rhesus or cynomolgus monkeys by Ficoll-hypaque density gradient centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) as described (Hartmann G, et al 1996 *Antisense Nucleic Acid Drug Dev* 6:231). Cells were suspended in RPMI 1640 culture medium supplemented with 10 % (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Gibco BRL, Grand Island, NY) (complete medium). Cells (final concentration 1×10^6 cells/ml) were cultured in complete medium in a 5 % CO₂ humidified incubator at 37°C. ODN and LPS (from *Salmonella typhimurium*, Sigma Chemical Co., St. Louis, MO) or anti-IgM were used as stimuli. For measurement of human NK lytic activity, PBMC were incubated at 5×10^6 /well in 24-well plates. Cultures were harvested after 24 hours, and cells were used as effectors in a standard 4 hours ⁵¹Cr-release assay against K562 target cells as previously described (Ballas Z. K., Rasmussen W. L., and Kriegl A. M. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* 157:1840; Ballas Z. K., and Rasmussen W. 1993. Lymphokine-activated killer cells. VII. IL-4 induces an NK1.1⁺ CD8 α^+ β^+ TCR- $\alpha\beta$ B220⁺ lymphokine-activated killer subset. *J Immunol* 150:17). For B cell proliferation, 1 μ Cl of ³H thymidine was added 18 hours before harvest, and the amount of ³H thymidine incorporation was determined by scintillation counting at day 5. Standard deviations of the triplicate wells were < 5%.

Flow cytometry on primate PBMC: Surface antigens on primate PBMC were stained as previously described (Hartmann G et al 1998 *J Pharmacol Exp Ther* 283:920). Monoclonal antibodies to CD3 (UCHL1), CD14 (M5E2), CD19 (B34), CD56 (B159), CD69 (FN50) and CD86 (231) (TUN-1) were purchased from Pharmingen, San Diego, CA. IgG₁ (MOPC-21) and IgG_{2a} (Hartmann G et al 1999 *PNAS* 96:9305) were used to control for non-specific staining. NK cells were identified by CD56 expression on CD3, CD14 and CD19 negative cells, whereas B cells were identified by expression of CD19. Flow cytometric data from 10000 cells per sample were acquired on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The viability of cells within the FSC/SSC gate used for analysis was examined by propidium iodide staining (2 μ g/ml) and found to be higher than 98 %. Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stanford, CA).

Immunization of chimpanzees and cynomolgus monkeys against HBsAg and evaluation of the humoral response: Fourteen cynomolgus monkeys (2.0-3.5 kg) were immunized with a pediatric dose of Engix-B (SmithKline Beecham Biologicals, Rixensart, BE) containing 10 μ g HBsAg adsorbed to alum (25 mg Al³⁺/mg HBsAg). This was administered alone (n=5), or combined with CpG ODN 1968 (n=5, 500 μ g) or CpG ODN 2006 (SEQ ID NO.: 246) (n=4, 150 μ g). Four chimpanzees (10-20 kg) were immunized in the same fashion with two receiving control vaccine (Engix-B only) and two receiving experimental vaccine (Engix-B plus 1 mg CpG ODN 2006). All vaccines were administered IM in the right anterior thigh in a total volume of 1 ml. Monkeys were maintained in the national facility of the Primate Research Center (Bogor, Indonesia) and chimpanzees were housed at Bioparc (Rockville, MD). Animals were monitored daily by animal care specialists. No symptoms of general ill health or local adverse reactions at the injection site were noted. Plasma was recovered by IV puncture prior to and at various times after immunization and was stored frozen (-20°C) until assayed for antibodies. Anti-HBs antibodies were detected using a commercial ELISA kit (Monoclonal Anti-HBs; Sanofi-Pasteur, Montreal, QC) and then were expressed in mIU/ml based on comparison with WHO defined standards (Monoclonal Anti-HBs Standard, Sanofi-Pasteur).

Results

Identification of CpG ODN with different profiles of *in vitro* immune activities:

Our studies showed that the precise bases on the 5' and 3' sides of a CpG dinucleotide within a CpG motif may have an impact on the level of immune activation of a synthetic ODN, but it has been unclear whether different CpG motifs might display different

immune effects. To evaluate this possibility, we tested a panel of CpG ODN for their ability to induce NK lytic activity, B cell proliferation, and to stimulate synthesis of TNF- α , IL-6, IFN- γ and IL-12 in murine spleen cells. Immunostimulatory activity of ODN without CpG motifs (ODN 1882 (SEQ ID NO.: 225), ODN 1983 (SEQ ID NO.: 226)) was negative or weak compared to CpG ODN. ODN with non optimal CpG motifs (ODN 1628 (SEQ ID NO.: 767), ODN 1758 (SEQ ID NO.: 1)) were less active than ODN containing CpG motifs flanked by two 5' pyrimidines and two 3' pyrimidines (ODN 1760 (SEQ ID NO.: 3), ODN 1826 (SEQ ID NO.: 69), ODN 1841 (SEQ ID NO.: 84)). ODN 1826 containing two optimal murine CpG motifs (5' GACGTT 3') (SEQ ID NO.: 1143) had the highest activity for 5 of 6 measured endpoints. Except for ODN 1628, all ODN showed a generally similar pattern of activity (NK cell-mediated lysis, B cell proliferation, IL-12, IL-6, TNF α , IFN- γ). Of note, ODN 1628, which was unique in this panel for containing two G-rich regions, showed preferential induction of IFN- γ synthesis but relatively low stimulation of the other activities.

Identification of *in vitro* assays which correlate with *in vivo* adjuvant activity:

Since adjuvant activity is an *in vivo* endpoint, we were interested in identifying *in vitro* assays that would predict the adjuvant activity of a CpG ODN *in vivo*. The same ODN used for *in vitro* endpoints therefore were tested for their adjuvant activity to immunize mice against HBsAg. This was carried out both with ODN alone and with ODN combined with alum, since earlier studies had shown strong synergy for CpG ODN and alum adjuvants (PCT Published Patent Application WO98/40100).

BALB/c mice immunized with HBsAg without adjuvant attained only low titers of anti-HBs by 4 weeks, and this was not affected by addition of control ODN. In contrast, addition of CpG ODN raised anti-HBs titers by 5 to 40 fold, depending on the sequence used. When alum was added, titers of anti HBs were approximately 6 times higher than with HBsAg alone. Specifically, control ODN had no effect and the various CpG ODN augmented these titers 2 to 36 fold. Results obtained with the different ODN

alone correlated very strongly ($r = 0.96$) with those obtained using the same ODN plus alum. When linear regression was performed, a very high degree of correlation was found between certain *in vitro* assays and *in vivo* augmentation of anti-HBs titers. Of all the *in vitro* endpoints examined, the induction of NK lytic activity showed the best correlation to *in vivo* adjuvant activity (without alum, $r = 0.98$; with alum, $r = 0.95$; $p < 0.0001$). A good correlation regarding adjuvant activity was also obtained for B-cell stimulation ($r = 0.84$ and 0.7), as well as secretion of TNF- α ($r = 0.9$ and 0.88), IL-12 ($r = 0.88$ and 0.86) and IL-6 ($r = 0.85$ and 0.91). The one *in vitro* assay that did not correlate well with the *in vivo* results was IFN- γ secretion ($r = 0.57$ and 0.68). These data demonstrate that *in vitro* assays for NK lytic activity, B cell activation and production of TNF- α , IL-6 and IL-12 provide valuable information *in vitro* to predict the adjuvant activity of a given ODN *in vivo*.

Screening of a phosphorothioate ODN panel to activate human NK cells: In previous studies we found that synthesis of inflammatory cytokines by human PBMC is induced by extremely low amounts of endotoxin (induced TNF- α secretion is detectable with just 6 pg/ml endotoxin, 2 logs more sensitive than murine immune cells). In contrast, activation of human B cells and induction of human NK cell lytic activity with endotoxin is low even at high endotoxin concentrations. Based on these results we selected activation of NK cells (lytic activity and CD59 expression) and B cells (proliferation and CD86 expression) as the most highly specific and reproducible assays with low inter-subject variability and used these assays for *in vitro* screening of a pool of ODN.

First we studied the effect of phosphorothioate ODN containing various combinations and permutations of CpG motifs on NK cell-mediated lysis of target cells. For clarity and ease of presentation, only data with selected representative CpG and control ODN are shown. Human PBMC were incubated with different phosphorothioate ODN (6 μ g/ml) for 24 hours and tested for their ability to lyse 51 Cr-labeled K562 cells. ODN with two 6-mer CpG motifs (either 5' GACGTT 3' (SEQ ID NO.: 1143) or 5' GTGCTT 3' (SEQ ID NO.: 1144)) in combination with a T γ C at the 5' end of the ODN (ODN 1840 5' TCCATGTGCTTCCTGCTT 3' (SEQ ID NO.: 83), ODN 1851 5' TCCGTGACGTTCTCTGACGTT 3' (SEQ ID NO.: 94) or with at least three 6-mer motifs without a T γ C at the 5' end (ODN 2013 (SEQ ID NO.: 253)) show intermediate activity.

High activity was found when the 5' Tpc directly preceded a 6-mer human CpG motif (5' TCGTCGTT 3' (SEQ ID NO.: 1145)) and was followed by two 6-mer motifs (ODN 2005 (SEQ ID NO.: 245), ODN 2006 (SEQ ID NO.: 246) and ODN 2007 (SEQ ID NO.: 247)). The best results were obtained when the 6-mer CpG motifs were separated from each other and from the 5' 8-mer CpG motif by Tpt (ODN 2006 (SEQ ID NO.: 246)).

Expression of the activation marker CD69 is rapidly upregulated on the surface of NK cells subsequent to stimulation. To confirm the results from the NK cell lysate assay, PBMC were incubated for 18 hours with ODN (2 µg/ml). CD69 expression was examined on CD56 positive NK cells (CD3, CD14 and CD19 negative). Although

induction of CD69 expression was less sequence restricted than stimulation of NK cell functional activity, control ODN (ODN 1982, ODN 2116, ODN 2117, ODN 2010)

showed only low activity similar to background levels. ODN with two human CpG

motifs separated by 5' TTTT 3' (ODN 1965 (SEQ ID NO.: 208)) or four human CpG motifs without spacing (ODN 2013 (SEQ ID NO.: 253)) were relatively more active at inducing CD69 expression than at stimulating NK cell lytic activity. Optimal NK cell

functional activity, as well as CD69 expression, was obtained with ODNs containing a Tpc dinucleotide preceding the human CpG motif, and additional human motifs within the sequence (ODN 2006 (SEQ ID NO.: 246), ODN 2007 (SEQ ID NO.: 247)).

Activity of phosphorothioate ODN for stimulating human B cells: In preliminary experiments we found that the percentage of proliferating B cells (CFSE assay, see

methods section) correlated with the surface expression of the co-stimulatory CD86 on B cells, as measured by flow cytometry. Thus we used CD86 expression on B cells to

screen a panel of ODN for their immunostimulatory activity. PBMC were incubated with 0.6 µg/ml ODN. Expression of CD86 (mean fluorescence intensity, MFI) was examined

on CD19 positive B cells. A poly C ODN (ODN 2017 (SEQ ID NO.: 257)) or ODN

without CpG dinucleotides (ODN 1982 (SEQ ID NO.: 225)) failed to stimulate human B

cells under these experimental conditions. A phosphorothioate ODN (ODN 2116 (SEQ ID NO.: 256)) with one optimal human CpG motif preceded by a Tpc (5' TCGTCGTT

3' (SEQ ID NO.: 1145)) had low activity. The presence of one human 6-mer CpG motif (5' GTCGTT 3' (SEQ ID NO.: 1144)) had no activating effect. Two of these CpG

motifs within the sequence showed no (ODN 1960 (SEQ ID NO.: 203), ODN 2016 (SEQ ID NO.: 256)) or intermediate (ODN 1965 (SEQ ID NO.: 208)) activity dependent on the

sequence context. If the ODN was composed of three or four copies of this motif (ODN 2012 (SEQ ID NO.: 252), ODN 2013 (SEQ ID NO.: 253), ODN 2014 (SEQ ID NO.: 254)), intermediate activity on B cells could be detected. The combination of the human 8-mer CpG motif on the 5' end of the ODN with two 6-mer CpG motifs (ODN 2005 (SEQ ID NO.: 245), ODN 2006 (SEQ ID NO.: 246), ODN 2007 (SEQ ID NO.: 247), ODN 2102 (SEQ ID NO.: 343), ODN 2103 (SEQ ID NO.: 344)) led to a considerable increase in the ability of the ODN to stimulate B cells. The spacing between the single motifs was critical. The separation of CpG motifs by Tpt was preferable (ODN 2006

(SEQ ID NO.: 246)) compared to unspaced CpG motifs (ODN 2005 (SEQ ID NO.: 245) also compare ODN 1965 (SEQ ID NO.: 208) to ODN 1960 (SEQ ID NO.: 203)). The

human 6-mer CpG motif (5' GTCGTT 3') was better than the optimal mouse 6-mer CpG motif (5' GACGTT 3' (SEQ ID NO.: 246)) when combined with the human 8-mer CpG

motif on the 5' end (ODN 2006 vs. ODN 2102 (SEQ ID NO.: 343) and ODN 2103 (SEQ ID NO.: 344)). A (TCG)₃ ODN was inactive or only weakly active, as were ODN

containing CpG dinucleotides flanked by guanines or other CpG dinucleotides (ODN 2010 (SEQ ID NO.: 250)). Taken together, the findings for NK cells and B cells showed

consistently that of the ODN tested, ODN 2006 (SEQ ID NO.: 246) has the highest immunostimulatory activity on human immune cells.

Comparative analysis of potency of CpG phosphorothioate ODN in different

primates: Different CpG motifs are optimal to activate murine and human immune cells.

Furthermore, the number and location of CpG motifs within an active phosphorothioate ODN is different in mice and humans. We were interested to know if CpG

phosphorothioate ODN show a similar activity among different species of primates. We compared a panel of CpG ODN for their ability to induce B cell proliferation in humans,

chimpanzees and rhesus or cynomolgus monkeys. The capability of ODN to stimulate

human B cell proliferation (Table 3) correlated well with their ability to induce CD86

expression on B cells. ODN 2006 (SEQ ID NO.: 246), which showed the highest activity in human B cells and NK cells, was also the most active in stimulating chimpanzees and

rhesus monkey B cell proliferation (Table 3). ODN 1968 (SEQ ID NO.: 211) and ODN 2006 (SEQ ID NO.: 246) gave the highest activation of cynomolgus monkey B-cells *in*

vitro (SI of 25 and 29 respectively at 6 µg ODN/ml). Surprisingly, CpG ODN 2007 (SEQ ID NO.: 247), which displayed similarly high activity as the optimal ODN 2006

(SEQ ID NO.: 246) in human cells, did not stimulate Rhesus monkey or chimpanzee B cell proliferation, and the ODN 1968 (SEQ ID NO.: 211) showed low activity. CpG ODN originally identified with high activity in mice (ODN 1760 (SEQ ID NO.: 3), ODN 1826 (SEQ ID NO.: 69)) showed little activity in monkeys (Table J).

Table J: Proliferative response of PBMC to phosphorothioate CpG ODN in primates

No addition	Humans	Chimpanzee	Rhesus monkey
	0.5 \pm 0.1	0.5 \pm 0.1	0.5 \pm 0.0
ODN 1760 (SEQ ID NO.: 3)	23 \pm 7	0.3 \pm 0.1	0.5 \pm 0.3
ODN 1826 (SEQ ID NO.: 69)	0.8 \pm 0.1	0.4 \pm 0.1	0.6 \pm 0.1
ODN 1968 (SEQ ID NO.: 211)	35 \pm 9	20.0 \pm 3.8	1.9 \pm 0.7
ODN 1982 (SEQ ID NO.: 225)	9.7 \pm 1.1	2.5 \pm 1.1	0.7 \pm 0.1
ODN 2006 (SEQ ID NO.: 246)	58 \pm 8	27.4 \pm 8.9	6.3 \pm 3.3
ODN 2007 (SEQ ID NO.: 247)	47 \pm 11	0.5 \pm 0.1	0.4 \pm 0.2

PBMC were prepared from peripheral blood and incubated with ODN (0.6 μ g/ml) as indicated for five days. Proliferation was measured by uptake of 3 H-thymidine (cpm/1000) during the last 18 hours. More than 95 % of proliferating cells were B-cells as determined using the CFSE assay. Four human probands, six chimpanzees and two rhesus monkeys were tested.

In vivo adjuvant activity of CpG ODN in chimpanzees and cynomolgus monkeys: In order to evaluate whether CpG ODN with strong *in vitro* stimulatory effects on primate cells had detectable adjuvant activity *in vivo*, cynomolgus monkeys and chimpanzees were immunized with Egerix B, which comprises HBsAg adsorbed to

alum, alone or with added ODN 1968 (500 μ g) or ODN 2006 (SEQ ID NO.: 246) (1 mg) respectively. Compared to controls not receiving CpG ODN, anti-HBs titers at 4 weeks post-prime and 2 weeks post-boost were 66- and 16-fold higher respectively in the monkeys, and 15- and 3-fold higher in the chimpanzees (Table K). Thus a clear adjuvant effect of CpG ODN was seen, and this was particularly striking after a single immunization.

Table K: Anti-HBs responses in primates immunized against HBsAg with CpG ODN¹

Primate species	n	CpG ODN	4 wks post-prime	2 wks post-boost
Cynomolgus monkey	5	None	15 \pm 44	4880 \pm 13113
	5	ODN 1968 (500 μ g) (SEQ ID NO. 211)	995 \pm 1309	76449 \pm 42094
Chimpanzee	2	None	6, 11	3712, 4706
	2	ODN 2006 (1 mg) (SEQ ID NO. 246)	125, 135	9640, 16800

¹Animals were immunized by IM injection of Egerix B containing 10 μ g HBsAg adsorbed to alum, alone or with added CpG ODN. Cynomolgus monkeys were boosted at 10 wks and chimpanzees were boosted at 4 wks post-prime. Anti-HBs was determined by ELISA assay; values for monkeys are GMT \pm SEM (n=5) whereas individual values for the two chimpanzees in each group are provided.

1 claim:

- 157 -

CLAIMS

1. A method of stimulating an immune response, comprising administering an immunostimulatory nucleic acid selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid, to a non-tolerant subject in an amount effective to induce an immune response in the non-tolerant subject.
2. The method of claim 1, wherein the immunostimulatory nucleic acid is a T-rich nucleic acid.
3. The method of claim 2, wherein the T-rich immunostimulatory nucleic acid is a poly T nucleic acid comprising
5' TTTT 3'.
4. The method of claim 3, wherein the poly T nucleic acid comprises
5' X₁ X₂TTT X₃ 3'
wherein X₁, X₂, X₃ and X₄ are nucleotides.
5. The method of claim 3, wherein the T-rich immunostimulatory nucleic acid comprises a plurality of poly T nucleic acid motifs.
6. The method of claim 4, wherein X₁X₂ is TT.
7. The method of claim 4, wherein X₃X₄ is TT.
8. The method of claim 4, wherein X₁X₂ is selected from the group consisting of TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, GT, GG, GA, and GC.
9. The method of claim 4, wherein X₃X₄ is selected from the group consisting of TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, GT, GG, GA, and GC.
10. The method of claim 3, wherein the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 25% T.
11. The method of claim 1, wherein the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 33% T.

- 158 -

12. The method of claim 1, wherein the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 40% T.
13. The method of claim 1, wherein the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 50% T.
14. The method of claim 1, wherein the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 60% T.
15. The method of claim 1, wherein the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 80% T.
16. The method of claim 1, wherein the immunostimulatory nucleic acid comprises at least 20 nucleotides.
17. The method of claim 1, wherein the immunostimulatory nucleic acid comprises at least 24 nucleotides.
18. The method of claim 1, wherein the immunostimulatory nucleic acid has a nucleotide backbone which includes at least one backbone modification.
19. The method of claim 18, wherein the backbone modification is a phosphorothioate modification.
20. The method of claim 18, wherein the nucleotide backbone is oligonucleic.
21. The method of claim 18, wherein the nucleotide backbone is entirely modified.
22. The method of claim 1, wherein the immunostimulatory nucleic acid is free of CpG dinucleotides.
23. The method of claim 1, wherein the immunostimulatory nucleic acid is free of unmodified CpG dinucleotides.
24. The method of claim 1, wherein the immunostimulatory nucleic acid is free of methylated CpG dinucleotides.

25. The method of claim 1, wherein the immunostimulatory nucleic acid is free of poly-C sequences.

26. The method of claim 1, wherein the immunostimulatory nucleic acid includes a poly-A sequence.

27. The method of claim 20, wherein the immunostimulatory nucleic acid includes a poly-Q sequence.

28. The method of claim 1, wherein the immunostimulatory nucleic acid comprises a nucleotide composition of greater than 25% C.

29. The method of claim 1, wherein the immunostimulatory nucleic acid comprises a nucleotide composition of greater than 25% A.

30. The method of claim 1, wherein the immunostimulatory nucleic acid is administered orally.

31. The method of claim 1, wherein the immunostimulatory nucleic acid is administered locally.

32. The method of claim 1, wherein the immunostimulatory nucleic acid is administered in a sustained release device.

33. The method of claim 1, wherein the immunostimulatory nucleic acid is administered mucosally to a mucosal surface.

34. The method of claim 33, wherein the immune response is a mucosal immune response.

35. The method of claim 33, wherein the immune response is a systemic immune response.

36. The method of claim 33, wherein the mucosal surface is selected from the group consisting of an oral, nasal, rectal, vaginal, and ocular surface.

37. The method of claim 1, further comprising exposing the subject to an antigen and wherein the immune response is an antigen-specific immune response.

38. The method of claim 37, wherein a nucleic acid vector which encodes the antigen is administered to the subject, and wherein the nucleic acid vector is separate from the immunostimulatory nucleic acid.

39. The method of claim 37, wherein the antigen is a peptide antigen.

40. The method of claim 1, further comprising isolating an immune cell from the subject, contacting the immune cell with an effective amount to activate the immune cell of the immunostimulatory nucleic acid and re-administering the activated immune cell to the subject.

41. The method of claim 40, wherein the immune cell is a leukocyte.

42. The method of claim 41, further comprising contacting the immune cell with an antigen.

43. The method of claim 40, wherein the antigen is selected from the group consisting of a tumor antigen, a viral antigen, a bacterial antigen, and a parasitic antigen.

44. The method of claim 40, wherein the immune cell is a dendritic cell.

45. The method of claim 1, wherein the subject has or is at risk of developing asthma and the method is a method of treating or preventing asthma in the subject.

46. The method of claim 1, wherein the subject has or is at risk of developing allergy and the method is a method of treating or preventing allergy.

47. The method of claim 1, wherein the subject has cancer and the method is a method of treating the cancer.

48. The method of claim 47, wherein the cancer is selected from the group consisting of biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; CNS cancer; colon cancer; connective tissue cancer; endometrial cancer; eye cancer; gastric cancer; intraepithelial neoplasms; larynx cancer; lymphomas; Hodgkin's lymphoma; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastoma; oral cancer; oral cavity cancer; ovarian cancer; pancreas

cancer, prostate cancer, rectal cancer, sarcomas, thyroid cancer, and renal cancer, as well as other carcinomas and sarcomas.

49. The method of claim 1, wherein the cancer is selected from the group consisting of bone cancer, brain and CNS cancer, connective tissue cancer, esophageal cancer, eye cancer, Hodgkin's lymphoma, larynx cancer, oral cavity cancer, skin cancer, and testicular cancer.

50. The method of claim 47, further comprising administering an anti-cancer therapy.

51. The method of claim 50, wherein the anti-cancer therapy is an antibody.

52. The method of claim 47, wherein the subject is a human.

53. The method of claim 47, wherein the subject is selected from the group consisting of a dog, a cat, and a horse.

54. The method of claim 1, further comprising administering an antibody specific for a cell surface antigen, and wherein the immune response results in antigen dependent cellular cytotoxicity (ADCC).

55. The method of claim 1, wherein the subject has or is at risk of developing an infectious disease and wherein the method is a method for treating or preventing the infectious disease.

56. The method of claim 54, wherein the subject is a human.

57. The method of claim 54, further comprising administering an antigen to the subject.

58. The method of claim 57, wherein the antigen is selected from the group consisting of a bacterial antigen, a viral antigen, a parasitic antigen, and a fungal antigen.

59. The method of claim 56, wherein the subject is selected from the group consisting of a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, and fish.

60. The method of claim 59, further comprising administering an antigen to the subject.

61. The method of claim 59, wherein the antigen is derived from a microorganism selected from the group consisting of herpesviridae, rotaviridae, orthomyxoviridae, toxoplasma, bacterophila, campylobacter, chlamidia, Rickettsia, and streptococcus.

62. The method of claim 1, wherein the immunostimulatory nucleic acid is a TG nucleic acid.

63. The method of claim 62, wherein the TG nucleic acid comprises



64. The method of claim 62, wherein the TG nucleic acid comprises



65. The method of claim 63, wherein N_1 is a nucleic acid sequence composed of a number of nucleotides ranging from (11- N_2) to (21- N_2).

66. The method of claim 63, wherein N_2 is a nucleic acid sequence composed of a number of nucleotides ranging from (11- N_1) to (21- N_1).

67. The method of claim 64, wherein N_1 is a nucleic acid sequence composed of a number of nucleotides ranging from (9- N_2) to (19- N_2).

68. The method of claim 64, wherein N_2 is a nucleic acid sequence composed of a number of nucleotides ranging from (9- N_1) to (19- N_1).

69. The method of claim 63, wherein X_2 is thymidine.

70. The method of claim 64, wherein X_3 is thymidine.

71. The method of claim 64, wherein X_1X_2 are nucleotides selected from the group consisting of GT, CG, GA, AA, AT, AG, CT, CA, CQ, TA and TT.

72. The method of claim 64, wherein X_3X_4 are nucleotides selected from the group consisting of TT, CT, AT, AG, CG, TC, AC, CC, TA, AA, and CA.

73. The method of claim 63, wherein X_1X_2 are nucleotides selected from the group consisting of TT, TC, TA and TG.

74. The method of claim 1, wherein the subject is at risk of developing cancer and the method is a method of preventing the cancer.

75. The method of claim 50, wherein the anti-cancer therapy is selected from the group consisting of a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine.

76. A method for preventing disease in a subject, comprising:
administering to the subject an immunostimulatory nucleic acid on a regular basis to prevent disease in the subject, wherein the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

77. A method for inducing an innate immune response, comprising
administering to the subject an immunostimulatory nucleic acid in an amount effective for activating an innate immune response, wherein the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

78. A composition comprising
a sustained release device including an immunostimulatory nucleic acid, wherein the immunostimulatory nucleic acid is free of unmethylated CpG motifs and is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

79. The composition of claim 78, wherein the immunostimulatory nucleic acid has a phosphodiester backbone.

80. A composition of a nutritional supplement comprising
an immunostimulatory nucleic acid in a delivery device selected from the group consisting of a capsule, a pill, and a sublingual tablet, wherein the immunostimulatory nucleic acid is free of unmethylated CpG motifs and is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

81. The composition of claim 80, wherein the immunostimulatory nucleic acid has a phosphorothioate backbone.

82. A composition comprising
an immunostimulatory nucleic acid and an antigen, wherein the immunostimulatory nucleic acid is free of unmethylated CpG motifs and is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

83. A composition comprising
an immunostimulatory nucleic acid and an anti-microbial agent, wherein the immunostimulatory nucleic acid is free of unmethylated CpG motifs and is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

84. The composition of claim 83, wherein the anti-microbial agent is selected from the group consisting of an anti-viral agent, an anti-fungal agent, an anti-parasitic agent, and an anti-bacterial agent.

85. The method of claim 5, wherein the immunostimulatory nucleic acid comprises at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 T motifs.

86. The method of claim 5, wherein at least 2 of the plurality of poly T motifs each comprises at least three contiguous T nucleotide residues.

87. The method of claim 5, wherein at least two of the poly T motifs each comprises at least four contiguous T nucleotide residues.

88. The method of claim 5, wherein the plurality of poly T motifs is at least 3 motifs and wherein at least 3 motifs each comprises at least 3 contiguous T nucleotide residues.

89. The method of claim 5, wherein the plurality of poly T motifs is at least 4 motifs and wherein the at least 4 motifs each comprises at least 3 contiguous T nucleotide residues.

90. The method of claim 5, wherein at least one of the plurality of poly T motifs comprises at least 5, at least 6, at least 7, or at least 8 contiguous nucleotide residues.

- 165 -

91. The method of claim 1, wherein the immunostimulatory nucleic acid is free of two CpG dinucleotides.

92. The method of claim 1, wherein the immunostimulatory nucleic acid is free of three CpG dinucleotides.

93. The method of claim 1, wherein the immunostimulatory nucleic acid includes at least two poly C sequences of at least 3 contiguous C nucleotide residues.

94. The method of claim 1, wherein the immunostimulatory nucleic acid is free of two poly A sequences of at least 3 contiguous A nucleotide residues.

95. A pharmaceutical composition comprising an effective amount for stimulating an immune response of an isolated immunostimulatory nucleic acid of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 32, 40, 41, 64, 65, 66, 67, 68, 69, 70, 71, 72, 85, 86, 87, 88, 89, 90, 91, 92, 93, or 94 and a pharmaceutically acceptable carrier.

96. A composition of matter, comprising an isolated immunostimulatory nucleic acid of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 32, 40, 41, 64, 65, 66, 67, 68, 69, 70, 71, 72, 85, 86, 87, 88, 89, 90, 91, 92, 93, or 94 and a pharmaceutically acceptable carrier.

97. The method of claim 80 wherein the nucleic acid further comprises a plurality of CpG motifs, and wherein the plurality is at least 3 motifs, at least 4 motifs and wherein the at least 4 motifs each comprises at least 3 contiguous T nucleotide residues.

98. The method of claim 90 wherein the plurality of CpG motifs and poly T motifs are interrupted.

99. A composition, comprising:
an immunostimulatory nucleic acid and an anti-cancer therapy,
formulated in a pharmaceutically-acceptable carrier and in an effective amount to treat a cancer or to reduce the risk of developing a cancer, wherein the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid and a TG nucleic acid.

100. A composition, comprising:

- 166 -

an immunostimulatory nucleic acid and an asthma/allergy medication,
formulated in a pharmaceutically-acceptable carrier and in an effective amount for preventing or treating an immune response associated with exposure to a mediator of asthma or allergy, wherein the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid.

101. A composition comprising

an immunostimulatory nucleic acid selected from the group consisting of SEQ ID NO: 95-136, SEQ ID NO: 138-152, SEQ ID NO: 154-222, SEQ ID NO: 224-245, SEQ ID NO: 247-261, SEQ ID NO: 263-299, SEQ ID NO: 301, SEQ ID NO: 303-4109, SEQ ID NO: 414-420, SEQ ID NO: 424, SEQ ID NO: 426-947, SEQ ID NO: 959-1022, SEQ ID NO: 1024-1093, and a pharmaceutically acceptable carrier.

102. A composition comprising

an immunostimulatory nucleic acid consisting essentially of:

5' M₁TCGTCGTTM₂3'

wherein at least one of the C₁ is unmethylated, wherein M₁ is a nucleic acid having at least one nucleotide, wherein M₂ is a nucleic acid having between 0 and 50 nucleotides, and wherein the immunostimulatory nucleic acid has less than 100 nucleotides.

103. A pharmaceutical composition comprising
an immunostimulatory nucleic acid comprising:

5' TCGTCGTT 3'

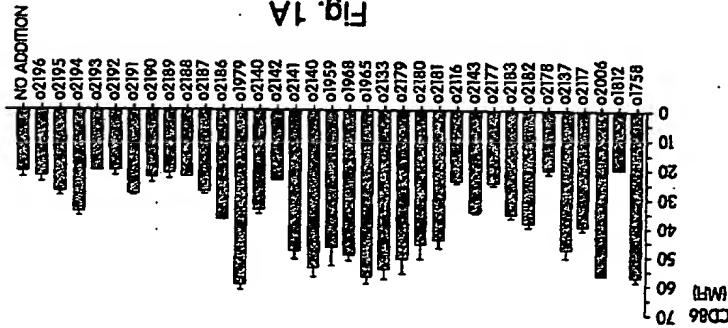
wherein at least one of the C₁ is unmethylated, wherein the immunostimulatory nucleic acid has less than 100 nucleotides and a phosphodiester backbone, and

a sustained release device.

104. The pharmaceutical composition of claim 103 wherein the sustained release device is a microparticle.

105. The pharmaceutical composition of claim 103, further comprising an antigen.

1/10



2/10

STATISTICAL ANALYSIS

	MEAN	STD DEV	STD ERROR	NUMBER	MINIMUM	MAXIMUM	# MISSING
01758	57.60	.71	.50	2	57.10	58.10	2
01812	20.70	.57	.40	2	20.30	21.10	2
02006	56.35	.35	.25	2	56.10	56.60	2
02117	40.00	1.13	.80	2	39.20	40.80	2
02137	47.60	3.39	2.40	2	45.20	50.00	2
02178	21.00	.99	.70	2	20.30	21.70	2
02182	37.75	.35	.25	2	37.50	38.00	2
02183	35.30	1.84	1.30	2	34.00	36.60	2
02177	25.00	.71	.50	2	24.50	25.50	2
02143	34.70	.71	.50	2	34.20	35.20	2
02116	24.35	.64	.45	2	23.90	24.80	2
02181	44.25	3.18	2.25	2	42.00	46.50	2
02180	45.90	5.94	4.20	2	41.70	50.10	2
02179	50.70	6.93	4.90	2	45.80	55.60	2
02133	53.75	4.31	3.05	2	50.70	56.80	2
01965	56.20	3.82	2.70	2	53.50	58.90	2
01968	49.35	1.91	1.35	2	48.00	50.70	2
02159	46.80	7.92	5.60	2	41.20	52.40	2
02140	53.25	4.74	3.35	2	49.90	56.60	2
02141	47.40	4.10	2.90	2	44.50	50.30	2
02142	23.20	.42	.30	2	22.90	23.50	2
01979	59.50	1.13	.80	2	58.30	60.70	2
02186	36.90	.14	.10	2	36.80	37.00	2
02187	27.15	.78	.55	2	26.60	27.70	2
02189	22.25	.21	.15	2	22.10	22.40	2
02190	22.95	1.20	.85	2	20.60	22.30	2
02191	28.35	.49	.35	2	28.00	28.70	2
02192	20.40	1.70	1.20	2	19.20	21.60	2
02193	19.70	.57	.40	2	19.30	20.10	2
02194	34.00	1.70	1.20	2	32.80	35.20	2
02195	27.30	1.27	.90	2	26.40	28.20	2
02196	22.45	2.76	1.95	2	20.50	24.40	2
NO ADDITION	20.90	2.77	1.38	4	18.60	24.90	0

Fig. 1B

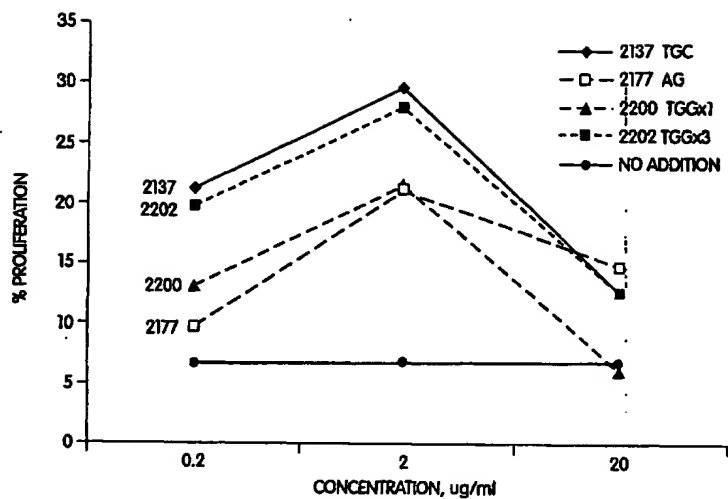


Fig. 2

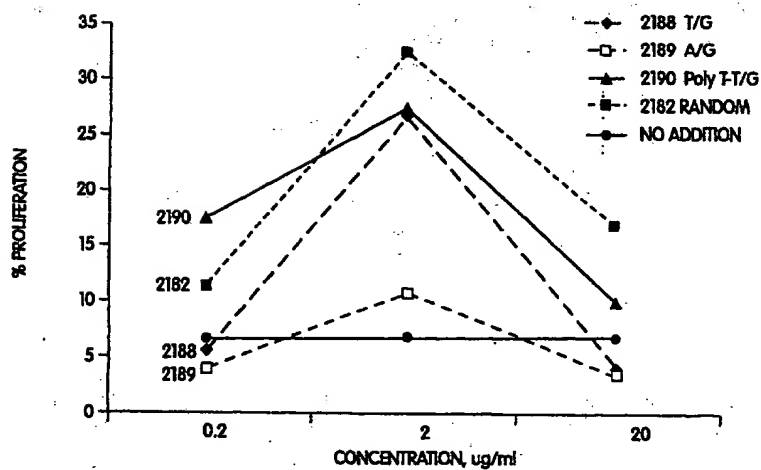


Fig. 3

WO 01/25973

PCT/US00/25383

5/10

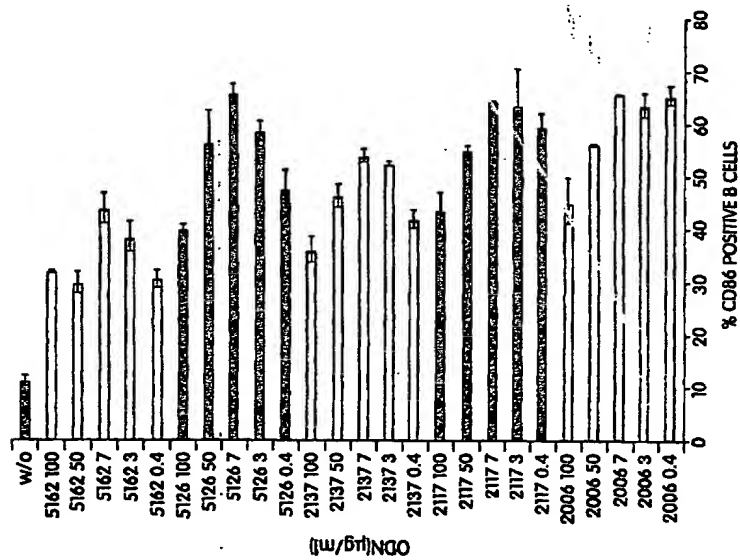


Fig. 4

SUBSTITUTE SHEET (RULE 26)

WO 01/25973

PCT/US00/25383

6/10

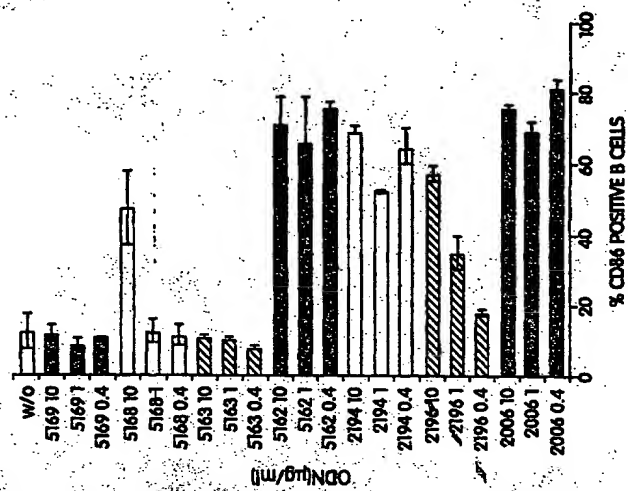


Fig. 5

SUBSTITUTE SHEET (RULE 26)

7/10

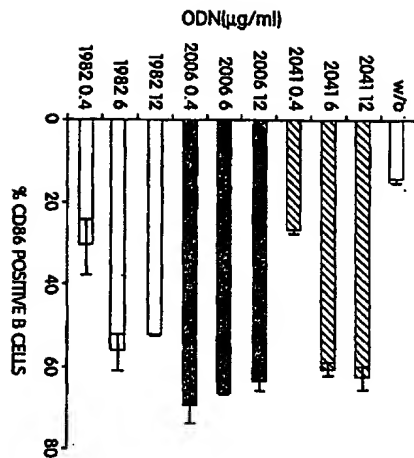


Fig. 6

8/10

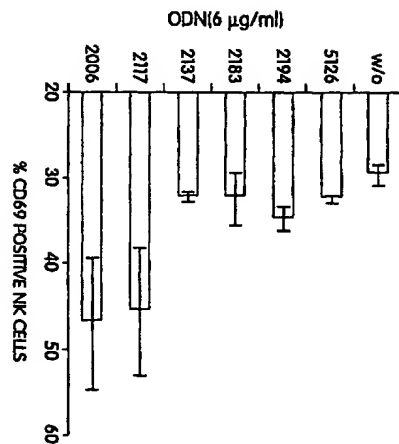


Fig. 7

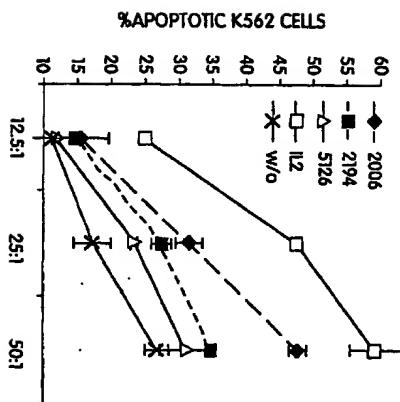


Fig. 8

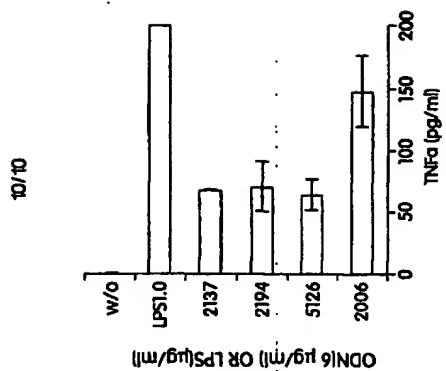


Fig. 11

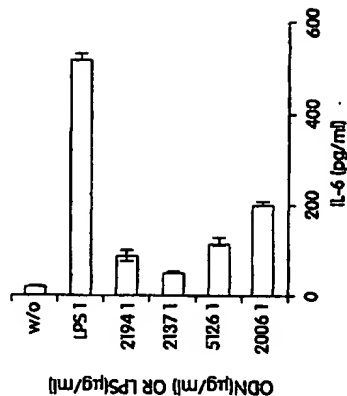


Fig. 12

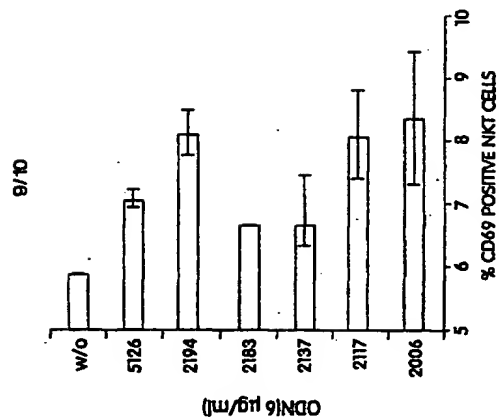


Fig. 9

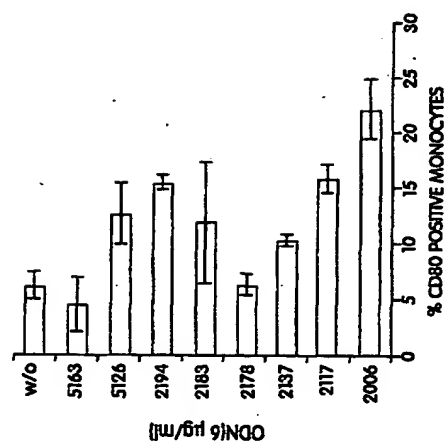


Fig. 10

WO 01/2972

PCT/US00/26383

1

SEQUENCE LISTING

<110> University of Iowa Research Foundation
Coley Pharmaceutical Group GmbH
<120> Immunostimulatory Nucleic Acids
<130> C1039/70390 (HCL/NAI)
<150> US 60/156,113
<151> 1999-09-25
<150> US 60/156,135
<151> 1999-09-27
<150> US 60/227,436
<151> 2000-08-23
<160> 1145
<170> FastSeq for Windows Version 3.0
<210> 1
<211> 18
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence
<400> 1
tctccagcgt tgcgcacat
18
<210> 2
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 2
ataatccagc ttgcacag
20
<210> 3
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 3
ataatccagc ttgcacag
20
<210> 4
<211> 18
<212> DNA
<213> Artificial Sequence
<400> 4
taccgcgtgc gccctctc
18
<210> 5
<211> 9
<212> DNA

WO 01/2972

PCT/US00/26383

2

<213> Artificial Sequence
<400> 5
ggggggggct
9
<210> 6
<211> 9
<212> DNA
<213> Artificial Sequence
<400> 6
gggggggggg
9
<210> 7
<211> 9
<212> DNA
<213> Artificial Sequence
<400> 7
ggcggggctg
9
<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified_base
<222> (8)...(8)
<223> m5c
<400> 8
tccatggtngt tccgtgatgc
20
<210> 9
<211> 15
<212> DNA
<213> Artificial Sequence
<220>
<221> modified_base
<222> (11)...(11)
<223> m5c
<400> 9
gtctactctg ngtga
15
<210> 10
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified_base
<222> (8)...(8)
<223> m5c
<400> 10
tccatggtngt tccgtgatgc
20
<210> 11

WO 91/21971

3

PCT/US90/03383

WO 91/21971

4

PCT/US90/03383

<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (13)...(13)
<223> m5c
<400> 11
tcacatgcgt tcmtgatgt
<210> 12
<211> 15
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (7)...(7)
<223> m5c
<400> 12
gctagangtt agtgt
<210> 13
<211> 19
<212> DNA
<213> Artificial Sequence
<400> 13
agctccatgg tgcctactg
<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 14
ccacgtgcgc cctcagccgc
<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 15
gcacatgctc ccgcagccgc
<210> 16
<211> 19
<212> DNA
<213> Artificial Sequence
<400> 16
gtcacctggt gcacatgctc
<210> 17
<211> 25
<212> DNA
<213> Artificial Sequence

<400> 17
gttggtatata ggcacagctt tgttg
<210> 18
<211> 23
<212> DNA
<213> Artificial Sequence
<400> 18
gcttcacact ggcctcactat taggc
<210> 19
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 19
accatggacy aactgtttcc cctc
<210> 20
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 20
accatggacy agctgtttcc cctc
<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 21
accatggacy accgttttcc cctc
<210> 22
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 22
accatggacy tactgtttcc cctc
<210> 23
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 23
accatggacy gtcgttttcc cctc
<210> 24
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 24
accatggacy ttctgtttcc cctc
<210> 25

25

25

24

24

24

24

24

24

WU 01/29/72

5

PCVUS00/26383

<211> 25
<212> DNA
<213> Artificial Sequence
<400> 25
ccatcacat ccgcctgcctcc acaag
<210> 26
<211> 25
<212> DNA
<213> Artificial Sequence
<400> 26
attctacata gccacctcttgcg tccag
<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 27
tccatgcgcct tccctgcgcct
<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 28
ggagagagagc agagagagagc
<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 29
ggagagagc tccgcgcgcct
<210> 30
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 30
aaaaaa
<210> 31
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 31
ccccc
<210> 32
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 32
ccgcgc
<210> 33
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 33
tccgcgc
<210> 34
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 34
tccgcgc
<210> 35
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 35
tccgcgc
<210> 36
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 36
tccgcgc
<210> 37

WU 01/29/72

6

PCVUS00/26383

<400> 29
ggagagagc tccgcgcgcct
<210> 30
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 30
aaaaaa
<210> 31
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 31
ccccc
<210> 32
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 32
ccgcgc
<210> 33
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 33
tccgcgc
<210> 34
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 34
tccgcgc
<210> 35
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 35
tccgcgc
<210> 36
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 36
tccgcgc
<210> 37

tcctatgaagt tcctgagttc	8	20
<210> 45		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 45		20
tcctatgaagt tcctgagttc		
<210> 46		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 46		20
tcctatgaagt tcctgagttc		
<210> 47		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 47		20
tcctatgaagt tcctgagttc		
<210> 48		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<220>		
<221> modified base		
<222> (8)... (8)		
<223> m5c		
<400> 48		20
tcctatgaagt tcctgagttc		
<210> 49		
<211> 39		
<212> DNA		
<213> Artificial Sequence		
<400> 49		39
tcctatgaagt tcctgagttc		
<210> 50		
<211> 39		
<212> DNA		
<213> Artificial Sequence		
<400> 50		39
tcctatgaagt tcctgagttc		
<210> 51		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 51		

tcctatgaagt tcctgagttc	8	20
<210> 45		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 45		20
tcctatgaagt tcctgagttc		
<210> 46		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 46		20
tcctatgaagt tcctgagttc		
<210> 47		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 47		20
tcctatgaagt tcctgagttc		
<210> 48		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<220>		
<221> modified base		
<222> (8)... (8)		
<223> m5c		
<400> 48		20
tcctatgaagt tcctgagttc		
<210> 49		
<211> 39		
<212> DNA		
<213> Artificial Sequence		
<400> 49		39
tcctatgaagt tcctgagttc		
<210> 50		
<211> 39		
<212> DNA		
<213> Artificial Sequence		
<400> 50		39
tcctatgaagt tcctgagttc		
<210> 51		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 51		

W0 01/29/72 PCT/US00/26383

9
20

cccccaggg atcgaaagct
<210> 52
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 52
agatagcaaa tccgctgacg
<210> 53
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 53
ggttcaggcg ctcattgctcg
<210> 54
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 54
tctccaggcg tgcgcacat
<210> 55
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 55
tctccaggcg tgcgcacat
<210> 56
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 56
taccgctgcg gacccctct
<210> 57
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 57
ataaccaggc tgcaccacag
<210> 58
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 58
ataaccaggc ttcacagcag
<210> 59
<211> 20
<212> DNA

W0 01/29/72 PCT/US00/26383

10

<213> Artificial Sequence
<400> 59
tccatgactt tcccgatttc
<210> 60
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 60
ttgatttttc gtttttttgc ttct
<210> 61
<211> 22
<212> DNA
<213> Artificial Sequence

<400> 61
tttttttgc ttttttgc tt
<210> 62
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 62
tgcctgacttc gttgctttgc gctt
<210> 63
<211> 22
<212> DNA
<213> Artificial Sequence

<400> 63
tgcctgacttc gtttcttgc tt
<210> 64
<211> 23
<212> DNA
<213> Artificial Sequence

<400> 64
gcattcacca ggcgggcag aat
<210> 65
<211> 23
<212> DNA
<213> Artificial Sequence

<400> 65
taccgagctt cgaagagatt tca
<210> 66
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 66
gcattgagctt gtagct
<210> 67
<211> 15

<212> DNA
<213> Artificial Sequence
<400> 82
gagggctgctct tcagggggg
<210> 83
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 83
tcctatgcctc tcctgcctct
<210> 84
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 84
tcctatgcctc tcctgcctct
<210> 85
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 85
tcctgcctctc ctcctctctc t
<210> 86
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 86
gcctgcctctc ggcctc
<210> 87
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 87
tcctccagcgc tgcgcctctc
<210> 88
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 88
tcctgcctctc tcctgcctctc
<210> 89
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 89
tcctccagcgc tgcgcctc
<210> 90
<211> 16
<212> DNA
<213> Artificial Sequence
<400> 90
tcctccagcgc ggcctc
<210> 91
<211> 18
<212> DNA
<213> Artificial Sequence
<400> 91
tcctccagcgc tgcgcctc
<210> 92
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 92
tcctgcctctc tcctgcctctc
<210> 93
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 93
gcctgcctctc ggcctc
<210> 94
<211> 19
<212> DNA
<213> Artificial Sequence
<400> 94
tcctgcctctc tcctgcctctc
<210> 95
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 95

tcctgcctctc tcctgcctctc
<210> 89
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 89
tcctgcctctc tcctgcctctc
<210> 90
<211> 16
<212> DNA
<213> Artificial Sequence
<400> 90
tcctccagcgc ggcctc
<210> 91
<211> 18
<212> DNA
<213> Artificial Sequence
<400> 91
tcctccagcgc tgcgcctc
<210> 92
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 92
tcctgcctctc tcctgcctctc
<210> 93
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 93
gcctgcctctc ggcctc
<210> 94
<211> 19
<212> DNA
<213> Artificial Sequence
<400> 94
tcctgcctctc tcctgcctctc
<210> 95
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 95

WO 01/23972	PCT/US90/26383	WO 01/23972	PCT/US90/26383
15	15	16	15
gcatgatgtt gacgt	<210> 96	<213> Artificial Sequence	<400> 103
<210> 96	<211> 15	gcatgagcgtt gacgt	<400> 104
<212> DNA	<213> Artificial Sequence	<210> 104	<211> 15
<213> Artificial Sequence	<400> 96	<212> DNA	<213> Artificial Sequence
gcatttcagag gacgt	<210> 97	<400> 104	gcatgagcgtt gacgt
<210> 97	<211> 15	<210> 105	<211> 15
<212> DNA	<213> Artificial Sequence	<212> DNA	<213> Artificial Sequence
<213> Artificial Sequence	<400> 97	<400> 105	gcatgagcgtt gacgt
gcatttcagcgtt gacgt	<210> 98	<210> 106	<211> 15
<210> 98	<211> 20	<212> DNA	<213> Artificial Sequence
<212> DNA	<213> Artificial Sequence	<400> 106	gcatgagcgtt gacgt
<213> Artificial Sequence	<400> 98	<210> 107	<211> 21
tccaggagcgtt tccagttcgt	<210> 99	<212> DNA	<213> Artificial Sequence
<210> 99	<211> 20	<400> 107	tccaggagcgtt tccagttcgt
<212> DNA	<213> Artificial Sequence	<210> 108	<211> 20
<213> Artificial Sequence	<400> 99	<212> DNA	<213> Artificial Sequence
tccaggagcgtt tccagttcgt	<210> 100	<400> 108	tccaggagcgtt tccagttcgt
<210> 100	<211> 20	<210> 109	<211> 20
<212> DNA	<213> Artificial Sequence	<212> DNA	<213> Artificial Sequence
<213> Artificial Sequence	<400> 100	<400> 109	tccaggagcgtt tccagttcgt
tccaggagcgtt tccagttcgt	<210> 101	<210> 110	<211> 20
<210> 101	<211> 20	<212> DNA	<213> Artificial Sequence
<212> DNA	<213> Artificial Sequence	<400> 110	tccaggagcgtt tccagttcgt
<213> Artificial Sequence	<400> 101	<210> 111	<211> 20
tccaggagcgtt tccagttcgt	<210> 102	<212> DNA	<213> Artificial Sequence
<210> 102	<211> 20	<400> 111	tccaggagcgtt tccagttcgt
<212> DNA	<213> Artificial Sequence	<210> 112	<211> 20
<213> Artificial Sequence	<400> 102	<212> DNA	<213> Artificial Sequence
tccaggagcgtt tccagttcgt	<210> 103	<400> 112	tccaggagcgtt tccagttcgt
<210> 103	<211> 15	<210> 113	<211> 20
<212> DNA	<213> Artificial Sequence	<212> DNA	<213> Artificial Sequence
<213> Artificial Sequence	<400> 103	<400> 113	tccaggagcgtt tccagttcgt

WO 01/2972	PCT/US90/26383	WO 01/2972	PCT/US90/26383
17	18		
<210> 111	<210> 117		
<211> 20	<211> 27		
<212> DNA	<212> DNA		
<213> Artificial Sequence	<213> Artificial Sequence		
<400> 111	<400> 117		
ttctctagcgt tgcgcacat	tcagacacg tgcgcggtg ttctctga		
20	27		
<210> 112	<210> 118		
<211> 20	<211> 27		
<212> DNA	<212> DNA		
<213> Artificial Sequence	<213> Artificial Sequence		
<400> 112	<400> 118		
tctccacagc tgcgccttc	tcagacacg ccgaccacgct gttcttga		
20	27		
<210> 113	<210> 119		
<211> 13	<211> 13		
<212> DNA	<212> DNA		
<213> Artificial Sequence	<213> Artificial Sequence		
<220> mlec_difference	<400> 119		
<221> mlec_difference	gctagtcgct agc		
<222> (5)...(5)	<210> 120		
<223> n la a or g or c or t/a	<211> 13		
<221> mlec_difference	<212> DNA		
<222> (6)...(6)	<213> Artificial Sequence		
<223> d la a or g or t/o/ not c	<400> 119		
<221> mlec_difference	gctagtcgct agc		
<222> (9)...(10)	<210> 120		
<223> h la a or c or t/o/ not g	<211> 13		
<400> 113	<212> DNA		
gcctandoghn agc	<213> Artificial Sequence		
13	13		
<210> 114	<400> 121		
<211> 11	gcttagcgtc tagc		
<212> DNA	<210> 122		
<213> Artificial Sequence	<211> 14		
<400> 114	<212> DNA		
tccttagcgtt ccc	<213> Artificial Sequence		
13	14		
<210> 115	<400> 122		
<211> 13	gcttagcgtt tagc		
<212> DNA	<210> 123		
<213> Artificial Sequence	<211> 14		
<400> 115	<212> DNA		
ggagagcgtt agc	<213> Artificial Sequence		
13	14		
<210> 116	<400> 123		
<211> 13	gcttagcgtc aagc		
<212> DNA	<210> 124		
<213> Artificial Sequence	<211> 14		
<400> 116	<212> DNA		
tccttagcgtt agc	<213> Artificial Sequence		
13	14		

WO 01/22972	PCT/US00/25353	WO 01/22972	PCT/US00/25353
19	14	20	24
<400> 124 gctagcgtt tagc		<212> DNA <213> Artificial Sequence	
<210> 125 <211> 20 <212> DNA <213> Artificial Sequence		<400> 132 ccgcttcctc cagatgagct catg	
<400> 125 tcctatgcct tccctgtct	20	<210> 133 <211> 24 <212> DNA <213> Artificial Sequence	
<210> 126 <211> 14 <212> DNA <213> Artificial Sequence		<400> 133 catgagctca tcagcagcga ggcg	24
<400> 126 gctagcgtc tagc	14	<210> 134 <211> 24 <212> DNA <213> Artificial Sequence	
<210> 127 <211> 19 <212> DNA <213> Artificial Sequence		<400> 134 ccagatgagc tcattgggtt ctcc	24
<400> 127 ggctatgctg tccatagcc	19	<210> 135 <211> 24 <212> DNA <213> Artificial Sequence	
<210> 128 <211> 19 <212> DNA <213> Artificial Sequence		<400> 135 ggagaacacc atgagctcat ctgg	24
<400> 128 ggctatgctg atcctagcc	19	<210> 136 <211> 20 <212> DNA <213> Artificial Sequence	
<210> 129 <211> 21 <212> DNA <213> Artificial Sequence		<400> 136 agctatcagg agcattgga	20
<400> 129 ctcatgggtt tctccaccac g	21	<210> 137 <211> 20 <212> DNA <213> Artificial Sequence	
<210> 130 <211> 21 <212> DNA <213> Artificial Sequence		<400> 137 tccatgacgt tccctgacgtt	20
<400> 130 cttgggtggg aahcccatga g	21	<210> 138 <211> 19 <212> DNA <213> Artificial Sequence	
<210> 131 <211> 20 <212> DNA <213> Artificial Sequence		<400> 139 gcgcgcgcgc gcgcgcgc	19
<400> 131 tccatgacgt tccctgtct	20	<210> 139 <211> 20 <212> DNA <213> Artificial Sequence	
<210> 132 <211> 24		<400> 139 ccgctctggc ggcgcgcgcg	20

WO 01/2972	21	PCT/US90/26383
<210> 140		
<211> 43		
<212> DNA		
<213> Artificial Sequence		
<400> 140		
ttccatccag cccacccgc ttggcccac ccctacact cca	43	
<210> 141		
<211> 43		
<212> DNA		
<213> Artificial Sequence		
<400> 141		
tgagggatga gggcggggc agagcgggtg gggcggatg gaa	43	
<210> 142		
<211> 27		
<212> DNA		
<213> Artificial Sequence		
<400> 142		
tcaatgtgg gatttccca tgaatct	27	
<210> 143		
<211> 27		
<212> DNA		
<213> Artificial Sequence		
<400> 143		
agactcatgg gaatatccca catttga	27	
<210> 144		
<211> 27		
<212> DNA		
<213> Artificial Sequence		
<400> 144		
tgccaaatgc tgaatcacta ataaaga	27	
<210> 145		
<211> 27		
<212> DNA		
<213> Artificial Sequence		
<400> 145		
tctttatgg tgaatcagca ctgtgca	27	
<210> 146		
<211> 31		
<212> DNA		
<213> Artificial Sequence		
<400> 146		
tgccaggaat ccgggttttc cccaccccca c	31	
<210> 147		
<211> 31		
<212> DNA		
<213> Artificial Sequence		

WO 01/2972	22	PCT/US90/26383
<400> 147		
ggggggttgg ggaataccg gacttctgc a	31	
<210> 148		
<211> 38		
<212> DNA		
<213> Artificial Sequence		
<400> 148		
ggggacttgc cgtcggggac ttccagggg gactttcc	38	
<210> 149		
<211> 45		
<212> DNA		
<213> Artificial Sequence		
<400> 149		
tccatggact tctcttccat gacgttcttc tccatggact tcttc	45	
<210> 150		
<211> 45		
<212> DNA		
<213> Artificial Sequence		
<400> 150		
gggaacgttc atggagagga acgtcatgga ggagaaatgc atcga	45	
<210> 151		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 151		
ataatagagc ttcaagaaag	20	
<210> 152		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 152		
tccatggact tccatggact	20	
<210> 153		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 153		
tccatggact tccatggact	20	
<210> 154		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 154		
tccaggaact tccatggact	20	
<210> 155		

<213> Artificial Sequence
<400> 170
tccctgacgtc gaagtc
15
<210> 171
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 171
tccctgacgtc gaagtc
15
<210> 172
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 172
tccctgacgtc gaagtc
15
<210> 173
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 173
gggggacgtc gggggg
15
<210> 174
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 174
tccctgacgtc ccttc
15
<210> 175
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 175
tccctgacgtc agagagagcgc at
22
<210> 176
<211> 32
<212> DNA
<213> Artificial Sequence
<400> 176
tccctgacgtc cccctgggcgc tccctctgcg ct
32
<210> 177
<211> 28
<212> DNA
<213> Artificial Sequence
<400> 177
tccctgacgtc cctctcgcctc cttctgcgt
28

<210> 178
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 178
tccctgacgtc gaagtc
15
<210> 179
<211> 15
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (1)...(7)
<223> m5c
<400> 179
tccctgacgtc gaagtc
15
<210> 180
<211> 15
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (3)...(3)
<223> m5c
<400> 180
tccctgacgtc gaagtc
15
<210> 181
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 181
tccctgacgtc gaagtc
15
<210> 182
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 182
tccctgacgtc gaagtc
15
<210> 183
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 183
tccctgacgtc gaagtc
15
<210> 184
<211> 15
<212> DNA

<213> Artificial Sequence		
<400> 184	15	
tccatgctgt gacgt		
<210> 185		
<211> 27		
<212> DNA		
<213> Artificial Sequence		
<400> 185	27	
ggctcgggg aggaatttt tgtctat		
<210> 186		
<211> 27		
<212> DNA		
<213> Artificial Sequence		
<400> 186	27	
atagacaaa ettcctccc cyggacc		
<210> 187		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 187	21	
tccatgctgt tccctggcto t		
<210> 188		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 188	21	
tccctgctgt ctacgtctct t		
<210> 189		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 189	21	
tctgtctgt ctccgtctct t		
<210> 190		
<211> 23		
<212> DNA		
<213> Artificial Sequence		
<400> 190	23	
tccgaactt gcaactcat ctg		
<210> 191		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 191	20	
caattatgc atgtctctgc		
<210> 192		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 192	20	
tccatgctgt tccatgctg		
<210> 193		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 193	20	
gcgatgctgt tccatgctg		
<210> 194		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 194	20	
gcgatgctgt tccatgctg		
<210> 195		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 195	20	
tccatgctgt tccatgctg		
<210> 196		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 196	20	
tccatgctgt tccatgctg		
<210> 197		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 197	20	
tccatgctgt tccatgctg		
<210> 198		
<211> 20		
<212> DNA		
<213> Artificial Sequence		
<400> 198	20	
gcggcgggg gcgcgcgcc		
<210> 199		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 199	20	
caattatgc atgtctctgc		

<210> 222
<211> 20
<212> DNA
<213> Artificial Sequence

21

<220>
<221> modified base
<222> (8)...(8)
<223> m5c

<221> modified base
<222> (17)...(17)
<223> m5c

<400> 222
tccatgtngt tccgtngtt

20

<210> 223
<211> 20
<212> DNA
<213> Artificial Sequence

15

<400> 223
tccatgacgt tccgacgtt

<210> 224
<211> 20
<212> DNA
<213> Artificial Sequence

20

<400> 224
gggggttgacg ttggggggg

<210> 225
<211> 20
<212> DNA
<213> Artificial Sequence

20

<400> 225
tccaggacgt cttcaggtt

<210> 226
<211> 20
<212> DNA
<213> Artificial Sequence

20

<400> 226
tttttttttt tttttttt

<210> 227
<211> 20
<212> DNA
<213> Artificial Sequence

20

<400> 227
tccatgacgt tccgacgtt

<210> 228
<211> 20
<212> DNA

20

<212> DNA
<213> Artificial Sequence

<400> 214
gggggtcgtc gtttggggg g

<210> 215
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 215
gggggtcgtc gtttggggg g

<210> 216
<211> 15
<212> DNA
<213> Artificial Sequence

21

<400> 216
tccggacgtt gacgt

<210> 217
<211> 15
<212> DNA
<213> Artificial Sequence

15

<400> 217
tccggacgtt gacgt

<210> 218
<211> 15
<212> DNA
<213> Artificial Sequence

15

<400> 218
tccggacgtt gacgt

<210> 219
<211> 15
<212> DNA
<213> Artificial Sequence

15

<400> 219
tccggacgtt gacgt

<210> 220
<211> 15
<212> DNA
<213> Artificial Sequence

15

<400> 220
tccggacgtt gacgt

<210> 221
<211> 15
<212> DNA
<213> Artificial Sequence

15

<400> 221
tccggacgtt gacgt

WO 01/21972
33
PCT/US00/26383
<213> Artificial Sequence
<400> 228
tccatgcgcg gcctgcgcgcg
20
<210> 229
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 229
tccatgcgcg tccctgcgcgt
20
<210> 230
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 230
tccatgcgcg tccctgcgcgcg
20
<210> 231
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 231
tccatgcgcg tccctgcgtt
20
<210> 232
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 232
tccatgcgcg tccctgcgcgt
20
<210> 233
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 233
tccatgcgcg cctgcgcgtt
20
<210> 234
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 234
tccatgcgtt gcctgcgtt
20
<210> 235
<211> 30
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)

WO 01/21972
34
PCT/US00/26383
<223> Biotin moiety attached at 5' end of sequence.
<400> 235
tccatccat tctagcgcg agctctccat
30
<210> 236
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 236
tccatgcgcg tccctgcgcgt
20
<210> 237
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 237
tccatgcgcg tccctgcgcgt
20
<210> 238
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 238
tccatgcgcg tccctgcgcgt
20
<210> 239
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 239
tccatgcgcg tccctgcgcgt
20
<210> 240
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 240
tccatgcgcg tccctgcgcgt
20
<210> 241
<211> 29
<212> DNA
<213> Artificial Sequence
<400> 241
tccatgcgtt tccctgcgcgt
29
<210> 242
<211> 29
<212> DNA
<213> Artificial Sequence
<400> 242
tccatgcgcg tccctgcgcgt
29

<21> modified base
<22> (10)...(10)
<23> msc
19
<21> modified base
<22> (13)...(13)
<23> msc
<400> 249
cggcgccggc gggcgccgg
<210> 250
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 250
gcgcgcggcg gcgcgcgcgc
<210> 251
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (3)...(3)
<223> I
<221> modified base
<222> (6)...(6)
<223> I
<221> modified base
<222> (14)...(14)
<223> I
<400> 251
agccgcggcg acgattccac
20
<210> 252
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 252
tgtcgtttgt cgttgcgtt t
21
<210> 253
<211> 25
<212> DNA
<213> Artificial Sequence
<400> 253
tgtcgttc grtgcgttg tgcgtt
25
<210> 254
<211> 25
<212> RNA
<213> Artificial Sequence

<210> 243
<211> 20
<212> DNA
<213> Artificial Sequence
20
<400> 243
ggcgccggcg ggcgcgcggc
<210> 244
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 244
tcacgcgcgt ttgcgcgtt
20
<210> 245
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 245
tgtcgtttgt cgttgcgtt
20
<210> 246
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 246
tgtcgttttt grtgcgtttgt cgtt
24
<210> 247
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 247
tgtcgtttgt cgttgcgtt tt
22
<210> 248
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 248
gcgtgcgttg tgcgttcgtt t
21
<210> 249
<211> 19
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (2)...(2)
<223> msc
<221> modified base
<222> (6)...(6)
<223> msc

W01/23/272	<100> 254 tctcgtctgc gcttcgtctgc tctgct
	<210> 255 <211> 14 <212> DNA <213> Artificial Sequence
	<400> 255 tctcgtcgtcgtc gcttc
	<210> 256 <211> 13 <212> DNA <213> Artificial Sequence
	<400> 256 tctcgtctgc gct
	<210> 257 <211> 13 <212> DNA <213> Artificial Sequence
	<400> 257 cccccacccc ccccacccc
	<210> 258 <211> 20 <212> DNA <213> Artificial Sequence
	<400> 258 tctcgtcgttc ttgcgtctcc
	<210> 259 <211> 20 <212> DNA <213> Artificial Sequence
	<400> 259 tgcacacccc aggcacacat
	<210> 260 <211> 23 <212> DNA <213> Artificial Sequence
	<400> 260 tctcgtcgttc cgtcgtcgtc gct
	<210> 261 <211> 20 <212> DNA <213> Artificial Sequence
	<400> 261 tctcgtcgttc gcttcgtcgtc
	<210> 262

WFO 01/23/97Z

38

<211> 24
<212> DNA
<213> Artificial Sequence

<100> 262

tcgccttcgtt gtccttcgtt cgtt

<210> 263
<211> 22
<212> DNA
<213> Artificial Sequence

<100> 263

tcgccttcgtt cgttccttcgtt tt

<210> 264
<211> 39
<212> DNA
<213> Artificial Sequence

<100> 264

gggggggggg gnaactctta aaattccccc agaatgctttt

<210> 265
<211> 39
<212> DNA
<213> Artificial Sequence

<100> 265

aaacattcttg ggggaatttt aagaattctc tccctccccc

<210> 266
<211> 33
<212> DNA
<213> Artificial Sequence

<100> 266

atgcttcactt cttaaaattc acccgaattg ttt

<210> 267
<211> 32
<212> DNA
<213> Artificial Sequence

<100> 267

aaacattcttg ggggaatttt aagaattctc cct

<210> 268
<211> 33
<212> DNA
<213> Artificial Sequence

<100> 268

atgcttcacta gcaaaaattc acccgaattg ttt

<210> 269
<211> 33
<212> DNA
<213> Artificial Sequence

<100> 269

aaacattctg ggggaatttt gcttagttaa
33

<210> 270

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 270

aaatttgag tttaaaaa

<210> 271

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 271

ccacttgacg ttttcoccc

<210> 272

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 272

ttttgtttgt tttgttgtt

<210> 273

<211> 24

<212> DNA

<213> Artificial Sequence

<400> 273

tgtgtgtttt gctgttttgt cgtt

<210> 274

<211> 14

<212> DNA

<213> Artificial Sequence

<400> 274

ctgcgcctg ggc

<210> 275

<211> 25

<212> DNA

<213> Artificial Sequence

<400> 275

ccctgtgta attatagtas aaccc

<210> 276

<211> 21

<212> DNA

<213> Artificial Sequence

<400> 276

ggtacactgt gggactttgt.g

<210> 277

<211> 18

<212> DNA
<213> Artificial Sequence

<400> 277

agccacgac gtgaggg

<210> 278

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 278

tccatgcggt tccgtgcggt

<210> 279

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 279

tccatgcggt tccgtgcggt

<210> 280

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 280

tccatgcggt tccgtgcggt

<210> 281

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 281

tccatgcggt tccgtgcggt

<210> 282

<211> 24

<212> DNA

<213> Artificial Sequence

<400> 282

ctggtctttt tggtttttt ctgg

<210> 283

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 283

tccgggggtg ggggaacctt

<210> 284

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<221> modified_base

WO 01/23972
<222> (8)...(8)
<223> m5c
<400> 284
tccatgaagt tccatgtctt
<210> 285
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 285
tccatgaagt tccatgtctt
<210> 286
<211> 26
<212> DNA
<213> Artificial Sequence
<400> 286
occgaagttca ttccctctca aacttgg
<210> 287
<211> 26
<212> DNA
<213> Artificial Sequence
<400> 287
ccaggttaag aggaatgac ttccggy
<210> 288
<211> 13
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (7)...(7)
<223> m5c
<400> 288
tccatggaag gaagt
<210> 289
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (2)...(2)
<223> m5c
<221> modified base
<222> (3)...(3)
<223> m5c
<231> modified base
<232> (9)...(9)
<233> m5c

41

PCT/US00/26383

WO 01/23972
<221> modified base
<222> (12)...(12)
<223> m5c
<221> modified base
<222> (14)...(14)
<223> m5c
<221> modified base
<222> (16)...(16)
<223> m5c
<400> 289
ggaggaggagg ggaggagccc
<210> 290
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 290
tccatgagct tccatgtctt
<210> 291
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 291
tccatgtctt tccatgtctt
<210> 292
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 292
tccatgagct tccatgtctt
<210> 293
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 293
tccatgagct tccatgtctt
<210> 294
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 294
tccatgagct tccatgtctt
<210> 295
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 295

42

PCT/US00/26383

<213> Artificial Sequence
 <400> 303
 tccaggactt ctctcaggtt
 <210> 304
 <211> 28
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (2)...(2)
 <223> m5c
 <221> modified base
 <222> (5)...(5)
 <223> m5c
 <221> modified base
 <222> (13)...(13)
 <223> m5c
 <221> modified base
 <222> (21)...(21)
 <223> m5c
 <400> 304
 tngtngtttt gtngttttgt nggt
 <210> 305
 <211> 29
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc feature
 <222> (1)...(3)
 <223> Biotin moiety attached at 5' end of sequence.
 <400> 305
 tctgtgtttt gtctgtttgt cgtttttt
 <210> 306
 <211> 18
 <212> DNA
 <213> Artificial Sequence
 <400> 306
 gctatgacgt tccaaagg
 <210> 307
 <211> 8
 <212> DNA
 <213> Artificial Sequence
 <400> 307
 tcaacggt
 <210> 308
 <211> 20

<213> Artificial Sequence
 <400> 296
 tctgtgaggt cctgtgaggt
 <210> 297
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (2)...(2)
 <223> m5c
 <221> modified base
 <222> (5)...(5)
 <223> m5c
 <221> modified base
 <222> (13)...(13)
 <223> m5c
 <221> modified base
 <222> (21)...(21)
 <223> m5c
 <400> 299
 tctgtgaggt gtagt
 <210> 300
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc feature
 <222> (1)...(3)
 <223> Biotin moiety attached at 5' end of sequence.
 <400> 301
 otgtgttttc tgtgtttttt ctgg
 <210> 302
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 302
 tcaatgacgt tctgtgaggt
 <210> 303
 <211> 20
 <212> DNA

WO 01/2972
45
PCT/US00/26383
<212> DNA
<213> Artificial Sequence
<400> 308
tccaggaact tccagagtt
<210> 309
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 309
ctctcgtgag gccacgttgg
<210> 310
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 310
cttccgcttg gccacgttgg
<210> 311
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 311
gtccggagca gcccaagtc
<210> 312
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 312
gtccggagca gcccaagtc
<210> 313
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 313
tccatgagtt tccatgagtt
<210> 314
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 314

WO 01/2972
46
PCT/US00/26383
aagagtcgcc ataccaaac
<210> 315
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 315
aagagtcgcc atccgcgggc
<210> 316
<211> 28
<212> DNA
<213> Artificial Sequence
<400> 316
<212> also difference
<222> (1)---(3)
<213> Protein moiety attached at 5' end of sequence.
ttttccatcg tggcttcctga tgcctttt
<210> 317
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 317
tccgtgcgtt gaagtttttt
<210> 318
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 318
gctagctctc ggcctcttga gctt
<210> 319
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 319
tgcgtcttc ccccccccc
<210> 320
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 320
tgcagcttcc ccccccccc
<210> 321
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 321

tctgtgttcc cccccccc	<210> 322	<213> Artificial Sequence	48	
	<211> 20			
	<212> DNA			15
	<213> Artificial Sequence			
tctgtgttcc cccccccc	<400> 322	<210> 330		
		<211> 21		
		<212> DNA		
		<213> Artificial Sequence		
tctgtgttcc cccccccc	<210> 323	<400> 330		21
	<211> 20	aaatctgtg cttttaaaaa n		
	<212> DNA	<210> 331		
	<213> Artificial Sequence	<211> 33		
		<212> DNA		
		<213> Artificial Sequence		
tctgtgttcc cccccccc	<400> 323	<400> 331		33
		gattccagttca cagtgcacgtg gcagatctg gat		
	<210> 324	<210> 332		
	<211> 20	<211> 33		
	<212> DNA	<212> DNA		
	<213> Artificial Sequence	<213> Artificial Sequence		
tctgtgttcc cccccccc	<400> 324	<400> 332		33
		gattccagtt cttgcacgtc actgtgactg gat		
	<210> 325	<210> 333		
	<211> 15	<211> 33		
	<212> DNA	<212> DNA		
	<213> Artificial Sequence	<213> Artificial Sequence		
tctgtgttcc cccccccc	<400> 325	<400> 333		33
		gattccagttca cagtgcacgtc gcagatctg gat		
	<210> 326	<210> 334		
	<211> 15	<211> 33		
	<212> DNA	<212> DNA		
	<213> Artificial Sequence	<213> Artificial Sequence		
tctgtgttcc cccccccc	<400> 326	<400> 334		33
		gattccagtt cttgcacgtc actgtgactg gat		
	<210> 327	<210> 335		
	<211> 15	<211> 20		
	<212> DNA	<212> DNA		
	<213> Artificial Sequence	<213> Artificial Sequence		
tctgtgttcc cccccccc	<400> 327	<220>		
		<221> modified base		
		<222> (16)...(16)		
		<223> m5c		
tctgtgttcc cccccccc	<210> 328	<400> 335		20
		tctgtgttcc cccccccc		
	<210> 329	<210> 336		
	<211> 15	<211> 20		
	<212> DNA	<212> DNA		

<213> Artificial Sequence
<220>
<221> modified base
<222> (2)...(2)
<223> m5c
<221> modified base
<222> (3)...(5)
<223> m5c
<400> 336
tngtngtgc cccccccc
20
<210> 337
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (2)...(2)
<223> m5c
<400> 337
tngtngtgc cccccccc
20
<210> 338
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (3)...(5)
<223> m5c
<400> 338
tngtngtgc cccccccc
20
<210> 339
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (3)...(5)
<223> m5c
<400> 339
tngtngtgc cccccccc
20
<210> 340
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (3)...(5)
<223> m5c
<400> 340
tngtngtgc cccccccc
20
<210> 341
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 341
tcggcgtgc cccccccc
20
<210> 342
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 342
ggcctttgc cccccccc
20
<210> 343
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 343
tcgtggttt gacgtttgc cgtt
24
<210> 344
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 344
tcgtggttt gacgtttgc cgtt
24
<210> 345
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 345
ccgtgcgtgc cccccccc
20
<210> 346
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 346
ggctgcgtgc cccccccc
20
<210> 347
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 347
tcgtcattgc cccccccc
20
<210> 348
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 348
acgtgcgtgc cccccccc
20
<210> 349
<211> 20

<223> m5c
 <400> 354
 tntgtgtttt gtcgtttttt cgtt
 <210> 355
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 355
 testgaggg gaagt
 <210> 356
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 356
 testgaaag gaagt
 <210> 357
 <211> 17
 <212> DNA
 <213> Artificial Sequence
 <400> 357
 testgtttt cccccc
 <210> 358
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (2)...(2)
 <223> m5c
 <221> modified base
 <222> (5)...(5)
 <223> m5c
 <221> modified base
 <222> (13)...(13)
 <223> m5c
 <221> modified base
 <222> (21)...(21)
 <223> m5c
 <400> 358
 tntgtgtttt gtcgtttttt ngct
 <210> 359
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 359
 ggggtcaagc tggagggggg

<212> DNA
 <213> Artificial Sequence
 <400> 349
 ctgtgtttt cccccccc
 <210> 350
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc_feature
 <222> (1)...(3)
 <223> Biotin moiety attached at 3' end of sequence.
 <400> 350
 tttttgttg tttccccccc cccc
 <210> 351
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc_feature
 <222> (1)...(24)
 <223> Biotin moiety attached at 3' end of sequence.
 <400> 351
 testgtttt cccccccc
 <210> 352
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc_feature
 <222> (22)...(24)
 <223> Biotin moiety attached at 3' end of sequence.
 <400> 352
 testgtttt gtcgtttttt cgtt
 <210> 353
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 353
 tcaagtttt testcgttt
 <210> 354
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (2)...(2)

W/O 01/25/97

53

PCT/US90/06383

<210> 360
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 360
tgcgtctcc cccccccc
<210> 361
<211> 14
<212> DNA
<213> Artificial Sequence
<400> 361
tcgtgcgcgt cgct
<210> 362
<211> 14
<212> DNA
<213> Artificial Sequence
<400> 362
tcgtgcgcgt cgct
<210> 363
<211> 14
<212> DNA
<213> Artificial Sequence
<400> 363
tcgtgcgcgt cgct
<210> 364
<211> 10
<212> DNA
<213> Artificial Sequence
<400> 364
tcaacgctga
<210> 365
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 365
tcaacgct
<210> 366
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 366
atggtttcc attttttac
<210> 367
<211> 20
<212> DNA
<213> Artificial Sequence

W/O 01/25/97

54

PCT/US90/06383

<400> 367
ataagtcgc atccgcgag
<210> 368
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 368
ataagtcgc atccgcgag
<210> 369
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 369
ataagtcgc atcccccac
<210> 370
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 370
tgctgcttt gtgcctttgc gatt
<210> 371
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 371
ctgtgcttc tgcgctttc tgcg
<210> 372
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 372
ctaactcttc taacttttt ctaa
<210> 373
<211> 26
<212> DNA
<213> Artificial Sequence
<400> 373
tcgtcgttcg tgcgttcgct
<210> 374
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 374
tcgtcgttcg tgcgttttc ggtc
<210> 375

WO 01/23971	PC7/US0076393	WO 01/23971	PC7/US0076393
55	24	56	23
<211> 24		cagacacaga agccctgatg acg	
<212> DNA			
<213> Artificial Sequence			
<400> 375	24	<210> 383	
acacatggacg agctgtttcc ctc		<211> 20	
		<212> DNA	
		<213> Artificial Sequence	
<210> 376		<400> 383	
<211> 20		agacagacac gaacgcgcg	
<212> DNA			
<213> Artificial Sequence		<210> 384	
		<211> 20	
<400> 376	20	<212> DNA	
tcgtcgtttt gctgcggttt		<213> Artificial Sequence	
		<400> 384	
<210> 377		gtctgtccca tgctctcgaa	
<211> 20			
<212> DNA		<210> 385	
<213> Artificial Sequence		<211> 20	
		<212> DNA	
<400> 377	20	<213> Artificial Sequence	
ctgtacagta gcttggagag		<400> 385	
		gctgtccacg ttactctccg	
<210> 378			
<211> 18		<210> 386	
<212> DNA		<211> 20	
<213> Artificial Sequence		<212> DNA	
		<213> Artificial Sequence	
<400> 378	18	<400> 386	
gagaaacgtg gacttcc		ggggctctca tacaacctgg g	
<210> 379		<210> 387	
<211> 20		<211> 18	
<212> DNA		<212> DNA	
<213> Artificial Sequence		<213> Artificial Sequence	
<400> 379	20	<400> 387	
cgggcagcto agctatctgg		ggggctccctg agactgcg	
<210> 380		<210> 388	
<211> 37		<211> 20	
<212> DNA		<212> DNA	
<213> Artificial Sequence		<213> Artificial Sequence	
<400> 380	37	<400> 388	
gtttccagat aaacgcggac cagaaacaga cacagaa		gagaaacgtg gacttccat	
<210> 381		<210> 389	
<211> 37		<211> 20	
<212> DNA		<212> DNA	
<213> Artificial Sequence		<213> Artificial Sequence	
<400> 381	37	<400> 389	
ttctgtgtct gtgtcgtggtt ccgtttatc tgaagaa		ttcatgtcgg tctgtatgt	
<210> 382		<210> 390	
<211> 23		<211> 20	
<212> DNA		<212> DNA	
<213> Artificial Sequence			
<400> 382			

W/O 01/29/72	57	PC10/S00/26383	W/O 01/29/72	58	PC10/S00/26383
<213> Artificial Sequence			<210> 398		
<400> 390			<211> 20		
ctctctggac ctggagagta	20		<212> DNA		
<210> 391			<213> Artificial Sequence		
<211> 20			<400> 398		
<212> DNA			gcacacacaaa ctgtccatg	20	
<213> Artificial Sequence			<210> 399		
<400> 391	20		<211> 20		
aggtacagcc agtactacga			<212> DNA		
<210> 392			<213> Artificial Sequence		
<211> 24			<400> 399		
<212> DNA			gtccatggcg tggggagatga	20	
<213> Artificial Sequence			<210> 400		
<400> 392	24		<211> 19		
acctatggagc acctgttccc cctc			<212> DNA		
<210> 393			<213> Artificial Sequence		
<211> 24			<400> 400		
<212> DNA			cctctatatac acctgggac	19	
<213> Artificial Sequence			<210> 401		
<400> 393	24		<211> 20		
acctatggatt accttttccc cctc			<212> DNA		
<210> 394			<213> Artificial Sequence		
<211> 20			<400> 401		
<212> DNA			cggcgagctc agtctatcgg	20	
<213> Artificial Sequence			<210> 402		
<400> 394	20		<211> 20		
atggaaagtc caagctctctc			<212> DNA		
<210> 395			<213> Artificial Sequence		
<211> 20			<400> 402		
<212> DNA			ggcttaccgg tagcctcagtc	20	
<213> Artificial Sequence			<210> 403		
<400> 395	20		<211> 35		
agcatcagga ccgaactggc			<212> DNA		
<210> 396			<213> Artificial Sequence		
<211> 20			<400> 403		
<212> DNA			cgactgcgca acagagatctc gttgtcagc acttgg	35	
<213> Artificial Sequence			<210> 404		
<400> 396	20		<211> 35		
ctctccaaagc tcaactcag			<212> DNA		
<210> 397			<213> Artificial Sequence		
<211> 21			<400> 404		
<212> DNA			ccagttgtctga tcaacagatat cctgtctggc agtctg	35	
<213> Artificial Sequence			<210> 405		
<400> 397	21		<211> 17		
tccctggagc tggccacct t			<212> DNA		
			<213> Artificial Sequence		

<212> DNA
<213> Artificial Sequence
<400> 413
tcgtggtttt gtcgttttgc cgtt

24

<210> 414
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_difference
<222> (3)...(3)
<223> n is a or c or g or t/u

<221> misc_difference
<222> (8)...(8)
<223> n is a or c or g or t/u

<221> misc_difference
<222> (11)...(11)
<223> n is a or c or g or t/u

<221> misc_difference
<222> (16)...(16)
<223> n is a or c or g or t/u

<221> misc_difference
<222> (19)...(19)
<223> n is a or c or g or t/u

<221> misc_difference
<222> (24)...(24)
<223> n is a or c or g or t/u

24

<400> 414
tcgtggtttt ntcgttttgc cgtt

<210> 415
<211> 17
<212> DNA
<213> Artificial Sequence

tctccacgcg tgcgcct

17

<210> 416
<211> 17
<212> DNA
<213> Artificial Sequence

tctccacgcg tgcgcct

17

<400> 416
<210> 417
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 417

<400> 405
ccgggttgta tgcgcgc
<210> 406
<211> 18
<212> DNA
<213> Artificial Sequence

17

tctccacgcg tgcgcct

18

<210> 407
<211> 18
<212> DNA
<213> Artificial Sequence

tctccacgcg tgcgcct

18

<210> 408
<211> 18
<212> DNA
<213> Artificial Sequence

tctccacgcg tgcgcct

18

<210> 409
<211> 18
<212> DNA
<213> Artificial Sequence

tctccacgcg tgcgcct

18

<210> 410
<211> 20
<212> DNA
<213> Artificial Sequence

ctatctgctg tctccacgcg

20

<210> 411
<211> 23
<212> DNA
<213> Artificial Sequence

tcgtggtttt gtcgttttgc cgt

23

<210> 412
<211> 24
<212> DNA
<213> Artificial Sequence

tcgtggtttt gtcgttttgc cgtt

24

<210> 413
<211> 24

WO 01/2972	61	PCT/US90/26393	WO 01/2972	62	PCT/US90/26393
ataatcgttc gttcaagaaa g	21		<213> Artificial Sequence		
<210> 418			<400> 425		19
<211> 20			ttcatgagcgt tccctgaccc		
<212> DNA			<210> 426		
<213> Artificial Sequence			<211> 21		
<400> 418		20	<212> DNA		
ataatcgaaggt tcccccctccc			<213> Artificial Sequence		
<210> 419			<400> 426		21
<211> 20			tcgtgcgtcgt tgcgtcttcc t		
<212> DNA			<210> 427		
<213> Artificial Sequence			<211> 24		
<400> 419		20	<212> DNA		
ttcatcgaaggt ttcagaagcag			<213> Artificial Sequence		
<210> 420			<400> 427		24
<211> 14			agagccttca gacctttaga gctt		
<212> DNA			<210> 428		
<213> Artificial Sequence			<211> 24		
<400> 420		14	<212> DNA		
ttcatgagcaggt gaagt			<213> Artificial Sequence		
<210> 421			<400> 428		24
<211> 19			cccccccccc ccccccccc cccc		
<212> DNA			<210> 429		
<213> Artificial Sequence			<211> 32		
<400> 421		19	<212> DNA		
ttcatgagcgt tccctgaccc			<213> Artificial Sequence		
<210> 422			<400> 429		32
<211> 19			tcgtgcgttct gtcgtcttgc cgttttgctg tt		
<212> DNA			<210> 430		
<213> Artificial Sequence			<211> 28		
<400> 422		19	<212> DNA		
ttcatgagcgt tccctgaccc			<213> Artificial Sequence		
<210> 423			<400> 430		28
<211> 19			tcgtgcgttt ttcgtcttct ttgtcgtt		
<212> DNA			<210> 431		
<213> Artificial Sequence			<211> 20		
<400> 423		19	<212> DNA		
ttcatgagcgt tccctgaccc			<213> Artificial Sequence		
<210> 424			<400> 431		20
<211> 15			tcgtgcgttct cttctttttt		
<212> DNA			<210> 432		
<213> Artificial Sequence			<211> 20		
<400> 424		15	<212> DNA		
tccttgagcgtg gaagt			<213> Artificial Sequence		
<210> 425			<400> 432		20
<211> 19			ttttttcaaggt ttgatttttt		
<212> DNA					

WO 01/2972	PCT/US00/26383	63	64	PCT/US00/26383
<210> 433 <211> 24 <212> DNA <213> Artificial Sequence				
tttttttttt tttttttttt tttt	24			
<400> 433 <210> 434 <211> 20 <212> DNA <213> Artificial Sequence				
gggggctgctg tttggggggg	20			
<400> 434 <210> 435 <211> 24 <212> DNA <213> Artificial Sequence				
tcgtcgtttt gtcgtttttg gggg	24			
<400> 435 <210> 436 <211> 27 <212> DNA <213> Artificial Sequence				
tcgtcgtgt ctcgcgtttct ttttgc	27			
<400> 436 <210> 437 <211> 15 <212> DNA <213> Artificial Sequence				
tcgtcgtgt atccg	15			
<400> 437 <210> 438 <211> 20 <212> DNA <213> Artificial Sequence				
ctgtaagta gcttggagag	20			
<400> 438 <210> 439 <211> 20 <212> DNA <213> Artificial Sequence				
gagaaagcty gaatttcatt	20			
<400> 439 <210> 440 <211> 17 <212> DNA <213> Artificial Sequence				
cccaacacga ggggaacgca	20			
<400> 440 ccaggttgta tagagc				
<210> 441 <211> 17 <212> DNA <213> Artificial Sequence				
gctagacgtt agctgca				
<400> 441 <210> 442 <211> 20 <212> DNA <213> Artificial Sequence				
ggagctcttc gaaagccata				
<400> 442 <210> 443 <211> 20 <212> DNA <213> Artificial Sequence				
tctccatgat ggttttatcg				
<400> 443 <210> 444 <211> 21 <212> DNA <213> Artificial Sequence				
aaggtggggc agtctcaagg a				
<400> 444 <210> 445 <211> 20 <212> DNA <213> Artificial Sequence				
atcggagagac tggcgcccg				
<400> 445 <210> 446 <211> 20 <212> DNA <213> Artificial Sequence				
ttaggacaaag gtcctayggty				
<400> 446 <210> 447 <211> 20 <212> DNA <213> Artificial Sequence				
acccacacga ggggaacgca				
<400> 447 <210> 448 <211> 20				

<212> DNA
<213> Artificial Sequence
<400> 448
gcacgctgcg gcacgcgcgg
<210> 449
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 449
gaacctcca tgcgtgt
<210> 450
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 450
gcacgcgcgt agcgtgca
<210> 451
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 451
gcctgcgcgc cctgcgcgcg
<210> 452
<211> 12
<212> DNA
<213> Artificial Sequence
<400> 452
gtgcctctcc ta
<210> 453
<211> 14
<212> DNA
<213> Artificial Sequence
<400> 453
cgtgcgcct ccca
<210> 454
<211> 16
<212> DNA
<213> Artificial Sequence
<400> 454
cacgtgcgc tctctta
<210> 455
<211> 18
<212> DNA
<213> Artificial Sequence
<400> 455
agcgcgcgtg cctctcta

<210> 456
<211> 18
<212> DNA
<213> Artificial Sequence
<400> 456
gaacgcgcga ccttccat
<210> 457
<211> 10
<212> DNA
<213> Artificial Sequence
<400> 457
gaacttccat
<210> 458
<211> 12
<212> DNA
<213> Artificial Sequence
<400> 458
tgcgcctccc at
<210> 459
<211> 14
<212> DNA
<213> Artificial Sequence
<400> 459
gcgcgcacct ccat
<210> 460
<211> 16
<212> DNA
<213> Artificial Sequence
<400> 460
acgcctgcgc cttccat
<210> 461
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 461
taagctctgt cacgcgcgcg
<210> 462
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 462
gcgcgcgcgc gacttccat gt
<210> 463
<211> 20
<212> DNA
<213> Artificial Sequence

<211> 20
 <212> DNA
 <213> Artificial Sequence
 tongtcttgg tacttttca
 <400> 471
 <210> 472
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 472
 tggttacggt cgtccactg
 <210> 473
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 473
 gtctatcgga ggaactgggc
 <210> 474
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 474
 cattttcagg gcgggcgggc
 <210> 475
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 475
 gagggagacca tttaacgggc
 <210> 476
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 476
 tgtccagccg agggaccat
 <210> 477
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 477
 cgggcttaac gggatgctg
 <210> 478
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 478

<400> 463
 tccatgtcgg tccgtatgct
 <210> 464
 <211> 21
 <212> DNA
 <213> Artificial Sequence
 <400> 464
 ttcatgctt gcaaatggc g
 <210> 465
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 465
 tgctagtctg gctgtgaact
 <210> 466
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 466
 agactcagga cagatcagga
 <210> 467
 <211> 22
 <212> DNA
 <213> Artificial Sequence
 <400> 467
 gaccttccat gtccgtctcg at
 <210> 468
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 468
 acacaccga gacggganc
 <210> 469
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 469
 gaaacttcca tgcgttcag
 <210> 470
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 470
 caatcaatct gggagagcgc
 <210> 471

WO 01/2972 PCT/US00/26383

69

20

tggaactct atgcgcgc

<210> 479

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 479

tggaactct tttagaagc

<210> 480

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 480

gtgttacgcg tgcgcacat

<210> 481

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 481

octcaaatcg aaagaccac

<210> 482

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 482

tigtaacttc catgttgtt

<210> 483

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 483

ttcaatgcgcg ttccgcgcgcg

<210> 484

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 484

gaactctat gtcgcgcgcg

<210> 485

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 485

gagagcgcgc gaactctgat

<210> 486

<211> 20

<212> DNA

20

WO 01/2972 PCT/US00/26383

70

<213> Artificial Sequence

<400> 486

ttgcaccata tttagaagac

<210> 487

<211> 18

<212> DNA

<213> Artificial Sequence

<400> 487

ttgaactcgc gttgcgcgc

<210> 488

<211> 21

<212> DNA

<213> Artificial Sequence

<400> 488

ctatcgagcg actgcgcgcgc c

<210> 489

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 489

cttgagagcg ctcgcgcgcgcg

<210> 490

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 490

gctgaactct ccatgcgtgtt

<210> 491

<211> 32

<212> DNA

<213> Artificial Sequence

<400> 491

tagaagcgcg attctctctt taggcgcgc ca

<210> 492

<211> 24

<212> DNA

<213> Artificial Sequence

<400> 492

agatggtctt cagataagc ggaac

<210> 493

<211> 24

<212> DNA

<213> Artificial Sequence

<400> 493

ttcgcgtcta tctgaagacc atctt

24

24

21

20

20

32

24

24

<400> 501
gggggttttca acgttttgag ggggg
<210> 502
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 502
ggttcagpta tgcacatat
<210> 503
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 503
ctgtcccata tttttgaca
<210> 504
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 504
accatctctga ggcatttcg
<210> 505
<211> 23
<212> DNA
<213> Artificial Sequence

<400> 505
cgtctatcgg gcttctgtgt atg
<210> 506
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 506
ggcctatccca catgaaagt t
<210> 507
<211> 22
<212> DNA
<213> Artificial Sequence

<400> 507
cctaatatcgy gtcgtcaagc ac
<210> 508
<211> 23
<212> DNA
<213> Artificial Sequence

<400> 508
gtgcttgaca accgatattt gg
<210> 509
<211> 26

<210> 494
<211> 23
<212> DNA
<213> Artificial Sequence

<400> 494
gtcccaaggtt gtagagggc tgc

<210> 495
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 495
gcgcctagtc tccgatagac

<210> 496
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 496
atcgggagac tggggcgccg

<210> 497
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 497
ggtctgtccc atatttttag

<210> 498
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 498
tttttcaacy ttgaaggggg

<210> 499
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 499
tttttcaagc gtgattttt t

<210> 500
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 500
gggggtcaacy ctgattttt

<210> 501
<211> 24
<212> DNA
<213> Artificial Sequence

W/O 01/29/72

73

PCV1500/2633

<212> DNA
<213> Artificial Sequence

<400> 509
gttgatgataca cagatctcat gttcgg

<210> 510
<211> 27
<212> DNA
<213> Artificial Sequence

<400> 510
gtccacacttc caatctgaga tggctctc

<210> 511
<211> 27
<212> DNA
<213> Artificial Sequence

<400> 511
ttcagcagaa tggctcagag atgtgtac

<210> 512
<211> 36
<212> DNA
<213> Artificial Sequence

<400> 512
tatagttcact ggaacttgaca caactctcca acaaac

<210> 513
<211> 27
<212> DNA
<213> Artificial Sequence

<400> 513
ggaagctctca taacactctgg gaactgga

<210> 514
<211> 22
<212> DNA
<213> Artificial Sequence

<400> 514
ctatcgagag aactgagcgc cg

<210> 515
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 515
tatcgagaga ctggcgccgc g

<210> 516
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 516
gatacgagaga ctggcgccgc g

26

27

27

36

27

27

21

21

W/O 01/29/72

74

PCV1500/2633

<210> 517
<211> 26
<212> DNA
<213> Artificial Sequence

<400> 517
ccgaacagga tatcggtgat caagac

<210> 518
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 518
tttcgggtgc aacgtctgaggg gggg

<210> 519
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 519
gggtcacagc tctgagggggg

<210> 520
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 520
cgcgcgcgcgc cgcgcgcgcgc

<210> 521
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 521
ggggcaatgac gttcggggggg

<210> 522
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 522
ggggcatcgac gttcaaaaaa

<210> 523
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 523
ggggcatcgag cttcggggggg

<210> 524
<211> 20
<212> DNA
<213> Artificial Sequence

26

24

20

20

20

20

20

20

<211> 20
<212> DNA
<213> Artificial Sequence

<400> 532
gctgttaaat gaatggcgcg
<210> 533
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 533
ttcgggggga ctcttcatt
<210> 534
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 534
tatgggcgcg ccggaattat
<210> 535
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 535
gggggtatcg atcagggggg
<210> 536
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 536
tttgagaag ctggacattc
<210> 537
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 537
gctcgtgat ctatgctcg
<210> 538
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 538
gtcgggtcctg atgctgttc
<210> 539
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 539

<400> 524
ggggcatgac gttcggggggg
<210> 525
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 525
aaaacatgac gttcaaaaa
<210> 526
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 526
aaaacatgac gttcggggggg
<210> 527
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 527
ggggcatgac gttcaaaaa
<210> 528
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 528
accatggag atctgtttcc octc
<210> 529
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 529
gacatggag aactgtttcc octc
<210> 530
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 530
cccccccccc ccccccccc
<210> 531
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 531
gggggggggg ggggggggg
<210> 532

WFO 01/23/97 77
 PCVUS00/263K3

ctgcctccag ttgcctctg
 <210> 540
 <211> 18
 <212> DNA
 <213> Artificial Sequence

ctggagcttc cagtcgag
 <400> 540
 <210> 541
 <211> 17
 <212> DNA
 <213> Artificial Sequence

gacgcctccag cgcctcct
 <400> 541
 <210> 542
 <211> 16
 <212> DNA
 <213> Artificial Sequence

ctggagcttc cagtcg
 <400> 542
 <210> 543
 <211> 16
 <212> DNA
 <213> Artificial Sequence

cactgcctcct cctcga
 <400> 543
 <210> 544
 <211> 18
 <212> DNA
 <213> Artificial Sequence

cgcctggagct tccactctgag
 <400> 544
 <210> 545
 <211> 20
 <212> DNA
 <213> Artificial Sequence

gctggagcttc ttgcctctgag
 <400> 545
 <210> 546
 <211> 20
 <212> DNA
 <213> Artificial Sequence

aacgcctggag cttccactctc
 <400> 546
 <210> 547
 <211> 20
 <212> DNA

WFO 01/23/97 78
 PCVUS00/263K3

<213> Artificial Sequence
 ttgcctgcctg aacagcctcct
 <400> 547
 <210> 548
 <211> 20
 <212> DNA
 <213> Artificial Sequence

cttccactcagt cgtgcctcctgac
 <400> 548
 <210> 549
 <211> 20
 <212> DNA
 <213> Artificial Sequence

ttactctctggag atccctctgag
 <400> 549
 <210> 550
 <211> 18
 <212> DNA
 <213> Artificial Sequence

ttccactctgag gtcctctgac
 <400> 550
 <210> 551
 <211> 18
 <212> DNA
 <213> Artificial Sequence

ctggatcgcctc cctcctgga
 <400> 551
 <210> 552
 <211> 20
 <212> DNA
 <213> Artificial Sequence

ggcgtctcctcct cctgcctcgc
 <400> 552
 <210> 553
 <211> 22
 <212> DNA
 <213> Artificial Sequence

ccttcactctgag atggcgcag ct
 <400> 553
 <210> 554
 <211> 20
 <212> DNA
 <213> Artificial Sequence

ggggtctactcag atggggggggg
 <400> 554
 <210> 554
 <211> 20

WO 01/25772	PCT/US00/25353	WO 01/25772	PCT/US00/25353
79		80	
<210> 553		<400> 562	
<211> 20		aaaaaccoco cccccaaaa	
<212> DNA		<210> 563	
<213> Artificial Sequence		<211> 27	
	20	<212> DNA	
<400> 555		<213> Artificial Sequence	
ttggggggg cctccacatt			27
<210> 556		<400> 563	
<211> 20		tttgaattca ggcgcgtga ggttgag	
<212> DNA		<210> 564	
<213> Artificial Sequence		<211> 27	
	20	<212> DNA	
<400> 556		<213> Artificial Sequence	
ttttttttt ttttttttt			27
<210> 557		<400> 564	
<211> 20		tttgaattct cagcgtctc cagtggc	
<212> DNA		<210> 565	
<213> Artificial Sequence		<211> 45	
	20	<212> DNA	
<400> 557		<213> Artificial Sequence	
gggggttttt ttttggggg			45
<210> 558		<400> 565	
<211> 20		aattctctct cggggcttct gtgtctgtg ctggttcgc tttat	
<212> DNA		<210> 566	
<213> Artificial Sequence		<211> 45	
	20	<212> DNA	
<400> 558		<213> Artificial Sequence	
ttttggggg ggggttttt			45
<210> 559		<400> 566	
<211> 19		ctagataag cggacccagc acagacaca gagcccccga tagag	
<212> DNA		<210> 567	
<213> Artificial Sequence		<211> 28	
	19	<212> DNA	
<400> 559		<213> Artificial Sequence	
ggggggggg ggggggggt			28
<210> 560		<400> 567	
<211> 20		ttttctagag agtgcacaa tgctctg	
<212> DNA		<210> 568	
<213> Artificial Sequence		<211> 29	
	20	<212> DNA	
<400> 560		<213> Artificial Sequence	
aaaaaaaaa aaaaaaaaa			29
<210> 561		<400> 568	
<211> 20		tttgaattcc gtgtacaga gcgcgaagc	
<212> DNA		<210> 569	
<213> Artificial Sequence		<211> 31	
	20	<212> DNA	
<400> 561		<213> Artificial Sequence	
cccccaaaa aaaaaccoco			31
<210> 562		<400> 569	
<211> 20		tttggcgcg ctgacttaa cctgagat a	
<212> DNA		<210> 570	
<213> Artificial Sequence		<211> 29	

WO 01/2972
81
PCT/US90/26383
<213> DNA
<213> Artificial Sequence
<400> 570
tttgggccc cgaagagcag agacacttc
<210> 571
<211> 29
<212> DNA
<213> Artificial Sequence
<400> 571
tttgggccc cttctcgctt cttgacacg
<210> 572
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 572
gagaaagctcg gaccttcacat
<210> 573
<211> 20
<212> DNA
<213> Artificial Sequence
tcctatgctcg tctctgatgct
<210> 574
<211> 6
<212> DNA
<213> Artificial Sequence
ctgtcg
<400> 574
<210> 575
<211> 6
<212> DNA
<213> Artificial Sequence
tcgtgga
<400> 575
<210> 576
<211> 6
<212> DNA
<213> Artificial Sequence
cgctcga
<400> 576
<210> 577
<211> 6
<212> DNA
<213> Artificial Sequence
atgtgct
<400> 577

WO 01/2972
82
PCT/US90/26383
<210> 578
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 578
ctgtcg
<210> 579
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 579
atgtgct
<210> 580
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 580
cgctcga
<210> 581
<211> 6
<212> DNA
<213> Artificial Sequence
tcgtgga
<400> 581
<210> 582
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 582
gagaaagctcg cagcttcgct
<210> 583
<211> 17
<212> DNA
<213> Artificial Sequence
gcttagagcga agcgtga
<400> 583
<210> 584
<211> 20
<212> DNA
<213> Artificial Sequence
gagaaagctcg gaccttcacat
<210> 585
<211> 21
<212> DNA
<213> Artificial Sequence

[illegible]

W0 01/29/97
85
PCT/US90/26383

atggaagctc cagctctctc
 <400> 599
 <210> 600
 <211> 6
 <212> DNA
 <213> Artificial Sequence
 <400> 600

tccctga
 <210> 601
 <211> 8
 <212> DNA
 <213> Artificial Sequence
 <400> 601

tcaagctt
 <210> 602
 <211> 8
 <212> DNA
 <213> Artificial Sequence
 <400> 602

aacgtt
 <210> 603
 <211> 8
 <212> DNA
 <213> Artificial Sequence
 <400> 603

aacgttga
 <210> 604
 <211> 17
 <212> DNA
 <213> Artificial Sequence
 <400> 604

tcaagctaac cctcagc
 <210> 605
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 605

ggaagctcgg gaccttgcac
 <210> 606
 <211> 14
 <212> DNA
 <213> Artificial Sequence
 <400> 606

gctggaacctt caat
 <210> 607

W0 01/29/97
86
PCT/US90/26383

ggaagctcgg gaccttgcac at
 <210> 608
 <211> 23
 <212> DNA
 <213> Artificial Sequence
 <400> 608

ggaagctcgg gaccttgcac cat
 <210> 609
 <211> 13
 <212> DNA
 <213> Artificial Sequence
 <400> 609

aacgttcagg ggcac
 <210> 610
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 610

atgcacctca aacgtt
 <210> 611
 <211> 10
 <212> DNA
 <213> Artificial Sequence
 <400> 611

tcaagcttga
 <210> 612
 <211> 14
 <212> DNA
 <213> Artificial Sequence
 <400> 612

gctggaacctt caat
 <210> 613
 <211> 7
 <212> DNA
 <213> Artificial Sequence
 <400> 613

caagctt
 <210> 614
 <211> 10
 <212> DNA
 <213> Artificial Sequence
 <400> 614

WO 01/2972	PCT/US0076383	87	88	PCT/US0076383
achacgttgc				
<210> 615				
<211> 6				
<212> DNA				
<213> Artificial Sequence				
<400> 615				
tcacgtt	6			
<210> 616				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 616				
tcacgatt	8			
<210> 617				
<211> 6				
<212> DNA				
<213> Artificial Sequence				
<400> 617				
tcagtc	6			
<210> 618				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 618				
aggtatc	8			
<210> 619				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 619				
tagagtc	8			
<210> 620				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 620				
gcgtcat	8			
<210> 621				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 621				
ccatgct	8			
<210> 622				
<211> 8				
<212> DNA				
gctgtgggc ggtactg	18			
atcgtgt				
<400> 622				
<210> 623				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 623				
atcgtgt				
<210> 624				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 624				
ccatgct				
<210> 625				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 625				
agcgtgt				
<210> 626				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 626				
tcacgtgt				
<210> 627				
<211> 8				
<212> DNA				
<213> Artificial Sequence				
<400> 627				
ccctgct				
<210> 628				
<211> 18				
<212> DNA				
<213> Artificial Sequence				
<400> 628				
gtgcgtgggt cccgggc				
<210> 629				
<211> 18				
<212> DNA				
<213> Artificial Sequence				
<400> 629				
gctgtgggc ggtactg				

<210> 630
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 630
tcaagct
<210> 631
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Fmoc moiety attached at 5' end of sequence.
<400> 631
tcaagct
<210> 632
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Fmoc moiety attached at 5' end of sequence.
<400> 632
aaagctga
<210> 633
<211> 7
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Fmoc moiety attached at 5' end of sequence.
<400> 633
tcaagct
<210> 634
<211> 7
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Fmoc moiety attached at 5' end of sequence.
<400> 634
aaagctg
<210> 635
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 635

cgaaga
<210> 636
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 636
tcaagct
<210> 637
<211> 5
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 637
tcgga
<210> 638
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 638
agaagct
<210> 639
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 639
tcaagct
<210> 640
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 640
tcaagct
<210> 641
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 641
caagct
<210> 642
<211> 6
<212> DNA
<213> Artificial Sequence
<220>
<221> misc_feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.
<400> 642
gtcga
<210> 643
<211> 6
<212> DNA
<213> Artificial Sequence
<400> 643

WO 01/25771

91

PCT/US90/2533

<21> Artificial Sequence

<400> 643

cgacgt

<210> 644

<211> 6

<212> DNA

<213> Artificial Sequence

<400> 644

oqtgt

<210> 645

<211> 6

<212> DNA

<213> Artificial Sequence

<400> 645

acgtgt

<210> 646

<211> 6

<212> DNA

<213> Artificial Sequence

<400> 646

cttgg

<210> 647

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 647

ggcagcgtg gacattcat

<210> 648

<211> 6

<212> DNA

<213> Artificial Sequence

<400> 648

ccgcta

<210> 649

<211> 6

<212> DNA

<213> Artificial Sequence

<400> 649

optacy

<210> 650

<211> 8

<212> DNA

<213> Artificial Sequence

<400> 650

tcacccgt

WO 01/25771

92

PCT/US90/2533

<210> 651

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 651

ccagcgatgc taccatgca

<210> 652

<211> 20

<212> DNA

<213> Artificial Sequence

<400> 652

accatcatmat agctctgtgc

<210> 653

<211> 8

<212> DNA

<213> Artificial Sequence

<400> 653

ccatcgat

<210> 654

<211> 8

<212> DNA

<213> Artificial Sequence

<400> 654

tcgacgtc

<210> 655

<211> 8

<212> DNA

<213> Artificial Sequence

<400> 655

ctagcgct

<210> 656

<211> 8

<212> DNA

<213> Artificial Sequence

<400> 656

taagcgct

<210> 657

<211> 13

<212> DNA

<213> Artificial Sequence

<400> 657

tcgcgaattc gcg

<210> 658

<211> 19

<212> DNA

<213> Artificial Sequence

<400> 658

tcgcgaattc gcg

<210> 659

<211> 13

<212> DNA

<213> Artificial Sequence

<400> 659

WFO 01/23/72
93
PCT/US80/25383

<400> 658
atggaaggct caagcttct
19
<210> 659
<211> 17
<212> DNA
<213> Artificial Sequence

<400> 659
actgaagctt agagttga
17
<210> 660
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 660
cgccctgggac tggctctgg
18
<210> 661
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 661
gtgtcggggt ctcggggc
18
<210> 662
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 662
gtgcggggt ctcggggc
18
<210> 663
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 663
cgccgtcgag ggggtctgg
18
<210> 664
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 664
gaagttcacg ttgagggaca t
21
<210> 665
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 665
atctgtgtgag ggcgaagctat g
21
<210> 666
<211> 21

WFO 01/23/72
94
PCT/US80/25383

<212> DNA
<213> Artificial Sequence

<400> 666
gttgaacac gagaaactca t
21
<210> 667
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 667
gcacagct
8
<210> 668
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 668
gtacagct
8
<210> 669
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 669
cgaaagct
8
<210> 670
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 670
gaaagct
8
<210> 671
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 671
caaaagct
8
<210> 672
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 672
ctaaagct
8
<210> 673
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 673
ggaagct
8
<210> 674
<211> 8
<212> DNA
<213> Artificial Sequence

WO 01/2572

PCT/US00/25333

95

<210> 674
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 674
tgaacgtt
<210> 675
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 675
acaacgtt
<210> 676
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 676
ttaacgtt
<210> 677
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 677
aaacgtt
<210> 678
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 678
ataacgtt
<210> 679
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 679
aacgttat
<210> 680
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 680
tcgaatcg
<210> 681
<211> 8
<212> DNA
<213> Artificial Sequence

WO 01/2572

PCT/US00/25333

96

<400> 681
tcgaatcg
<210> 682
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 682
gctagaagctt agagctga
<210> 683
<211> 25
<212> DNA
<213> Artificial Sequence
<400> 683
gagaaacgttg gaactcatca tccat
<210> 684
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 684
gagaaacgtta gaactcttat
<210> 685
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 685
actagaacgtt agtggga
<210> 686
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 686
cacacacttgg tcaatgtcaac gt
<210> 687
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 687
tctcatctct atgtttttat cg
<210> 688
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 688
cagatggacat tccat
<210> 689

15

22

17

20

25

17

8

<211> 23
<212> DNA
<213> Artificial Sequence
<400> 689
cacacacttg gtcacttgcg cgt
<210> 690
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 690
gtcagagcgt agctgcga
<210> 691
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 691
atgagagctg cagatcag
<210> 692
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 692
ctttgcgttt gtgcgtttcgt ggtc
<210> 693
<211> 23
<212> DNA
<213> Artificial Sequence
<400> 693
ctttgcgtttg tctgtttgtc ggt
<210> 694
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 694
ctttgcgtttcgt gtgcgt
<210> 695
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 695
aacgagctgga ttcagagcca
<210> 696
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 696

23

17

17

24

23

24

20

gtcagagcgt agcgt
<210> 697
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 697
aacgtcgaa cttccat
<210> 698
<211> 8
<212> DNA
<213> Artificial Sequence
<220>
<221> modified base
<222> (3)....(3)
<223> mc
<400> 698
tcagaggt
<210> 699
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 699
cccttcgat
<210> 700
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 700
actagagcgt agctgcga
<210> 701
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 701
gtcagagcgt agcgtgcga
<210> 702
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 702
atgagactcgc cagcttctc
<210> 703
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 703

17

8

8

17

17

20

WO 01/2373

PCT/US00/26383

99

atcgactctc gagctttctc

20

<210> 704
<211> 13
<212> DNA
<213> Artificial Sequence

<400> 704

gctagacgtt agc

13

<210> 705
<211> 9
<212> DNA
<213> Artificial Sequence

<400> 705

gctagacgtt

9

<210> 706
<211> 17
<212> DNA
<213> Artificial Sequence

<400> 706

agtcgacgtc gagatcg

17

<210> 707
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 707

ttacgacgtt
<220> modified base
<221> (3)...(5)
<222> (3)...(5)
<223> m5c

<400> 707

ttacgacgtt

8

<210> 708
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 708

atgatttctc tctcgtga

18

<210> 709
<211> 8
<212> DNA
<213> Artificial Sequence

<400> 709

<220> modified base
<221> (2)...(2)
<222> (2)...(2)
<223> m5c

<400> 709

ttacgacgtt

8

<210> 710

WO 01/2373

PCT/US00/26383

100

<211> 20
<212> DNA
<213> Artificial Sequence

<220> modified base
<221> (6)...(6)
<222> (6)...(6)
<223> m5c

<400> 710

gagacgctg gaccttcctc

20

<210> 711
<211> 17
<212> DNA
<213> Artificial Sequence

<400> 711

gctagacgtt agcttga

17

<210> 712
<211> 14
<212> DNA
<213> Artificial Sequence

<400> 712

gctaccttgc gtcga

14

<210> 713
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 713

gctaccttgc cgtga

15

<210> 714
<211> 19
<212> DNA
<213> Artificial Sequence

<400> 714

atcgactctg agctttctc

19

<210> 715
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 715

atcgactctg cagctttctc

20

<210> 716
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 716

agtcgactctc cagctttctc

20

<210> 717

W0 01/29/72
101
PCT/US00/26383

<211> 17
<212> DNA
<213> Artificial Sequence

gacagagctc agctcga
<400> 717
<210> 718
<211> 18
<212> DNA
<213> Artificial Sequence

atcgactcga gctcttc
<400> 718
<210> 719
<211> 17
<212> DNA
<213> Artificial Sequence

atcgatcgaag gctcttc
<400> 719
<210> 720
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> misc. feature
<222> (1)...(3)
<223> Biotin moiety attached at 5' end of sequence.

gagagcgcgc gacttcgat
<400> 720
<210> 721
<211> 17
<212> DNA
<213> Artificial Sequence

gctcagagctc agctcga
<400> 721
<210> 722
<211> 20
<212> DNA
<213> Artificial Sequence

atcgactcga gacttcgc
<400> 722
<210> 723
<211> 15
<212> DNA
<213> Artificial Sequence

tcagagctcag cgtcga
<400> 723
<210> 724

W0 01/29/72
102
PCT/US00/26383

<211> 18
<212> DNA
<213> Artificial Sequence

cgacttcga gctcttc
<400> 724
<210> 725
<211> 21
<212> DNA
<213> Artificial Sequence

ggggtcgaacc tcgagggggg g
<400> 725
<210> 726
<211> 16
<212> DNA
<213> Artificial Sequence

gctcagcttc gctcga
<400> 726
<210> 727
<211> 9
<212> DNA
<213> Artificial Sequence

cgtcgcgcgc
<400> 727
<210> 728
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> modified base
<222> (14)...(14)
<223> m5c

gagagcgcgc gacttcgat
<400> 728
<210> 729
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> modified base
<222> (18)...(18)
<223> m5c

atcgactcga gctcttcgc
<400> 729
<210> 730
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (3)...(3)
<223> m5c
20
<400> 730
atgaacaaat gtcgtttctc
<210> 731
<211> 15
<212> DNA
<213> Artificial Sequence
<220>
<221> modified_base
<222> (7)...(7)
<223> m5c
15
<400> 731
gttagangtt agcgt
<210> 732
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<221> modified_base
<222> (14)...(14)
<223> m5c
20
<400> 732
atagactctc gacgtttctc
<210> 733
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 733
gggtgtatgc atcagggggg
<210> 734
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 734
ggctgtatgc ctgactgccc
<210> 735
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 735
ccatgtatac ctctaga
<210> 736
<211> 17

<212> DNA
<213> Artificial Sequence
<400> 736
gctagatggt agcgtga
17
<210> 737
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 737
cgtacattca ggtga
15
<210> 738
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 738
tccatgtcgg tccatgtcgt
20
<210> 739
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 739
atcgactctc tccagcttc to
22
<210> 740
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 740
gctagatggt agcgtga
17
<210> 741
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 741
atcgactctc gactgtctc
20
<210> 742
<211> 17
<212> DNA
<213> Artificial Sequence
<400> 742
aacgctctgac cttagat
17
<210> 743
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 743
ctcaacgcty gacctccat
20

W0 01/29/72

105

PCVUS00/26383

<210> 744
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 744
atcgactac ggcactctc

<210> 745
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 745
ggagatgctg gacttcac

<210> 746
<211> 17
<212> DNA
<213> Artificial Sequence

<400> 746
tcaagctaac cctcgaac

<210> 747
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> misc. feature
<222> (1)...(3)
<223> Nucleotide moiety attached at 5' end of sequence.

<400> 747
ggagacgctc cagcactgat

<210> 748
<211> 20
<212> DNA
<213> Artificial Sequence

<230>
<231> misc. feature
<232> (1)...(3)
<233> Nucleotide moiety attached at 5' end of sequence.

<400> 748
ggagacgctg gacttcac

<210> 749
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 749
cgctagagct tgccttga

<210> 750
<211> 15

W0 01/29/72

106

PCVUS00/26383

<212> DNA
<213> Artificial Sequence

<400> 750
gctagagctc aagct

<210> 751
<211> 19
<212> DNA
<213> Artificial Sequence

<400> 751
atggaagctc cagctcttc

<210> 752
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 752
gcttagagctc aagct

<210> 753
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 753
gcttagagctc aagct

<210> 754
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 754
tccatgagctg tccctgatgct

<210> 755
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 755
tccatgagctg tccctgatgct

<210> 756
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 756
gcttagagctc aagct

<210> 757
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 757
gcttagagctc aagct

<210> 758
<211> 15

<400> 763
 tccatgtcgt tctgtatgct
 <210> 764
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (3)...(3)
 <223> m5c
 <220>
 <221> modified base
 <222> (10)...(10)
 <223> m5c
 <220>
 <221> modified base
 <222> (14)...(14)
 <223> m5c
 <400> 764
 atnqactctn ggggtttctc
 <210> 765
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 765
 atggaggttc cagttgtctc
 <210> 766
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 766
 gcatgacgtt ggcgt
 <210> 767
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 767
 ggggttcacg ttgggggggg
 <210> 768
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 768
 ggggttcacgt ctgggggggg
 <210> 769
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<210> 759
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 758
 tccatgacgt tctgtatgct
 <210> 759
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 759
 tccatgacgt tctgtatgct
 <210> 760
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (13)...(13)
 <223> m5c
 <400> 760
 gctagacgtt agcgt
 <210> 761
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 761
 gctagacgtt agcgt
 <210> 762
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (8)...(8)
 <223> m5c
 <400> 762
 tccatgtcgt tccatgtcgt
 <210> 763
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (12)...(12)
 <223> m5c

	W0	b1z797	
cgcgcygcgc gcgcggcgccg	<10>- 769		
<210> 770			
<211> 28			
<212> DNA			
<213> Artificial Sequence			
<100> 770			
cccccccacc ccccctccc acccacc			
<210> 771			
<211> 35			
<212> DNA			
<213> Artificial Sequence			
<100> 771			
cccccccccac ccgcccccac gcccca			
<210> 772			
<211> 20			
<212> DNA			
<213> Artificial Sequence			
<100> 772			
tccatgttcgc tccgtgatcct			
<210> 773			
<211> 15			
<212> DNA			
<213> Artificial Sequence			
<100> 773			
gtcaaaagct agacct			
<210> 774			
<211> 20			
<212> DNA			
<213> Artificial Sequence			
<100> 774			
tccatgttcga tccgtgatcct			
<210> 775			
<211> 20			
<212> DNA			
<213> Artificial Sequence			
<100> 775			
tccatgcgcgc tccttgatcct			
<210> 776			
<211> 20			
<212> DNA			
<213> Artificial Sequence			
<100> 776			
aasactcacagg ttcaaataaaa			
<210> 777			
<211> 20			

WFO 01/29/72	110	PCY0500/2533
<212> DNA		
<213> Artificial Sequence		
<400> 777		
tcctaaagct tccgtagct		
<210> 778		
<211> 23		
<212> DNA		
<213> Artificial Sequence		
<400> 778		
tcgaagctccc accgaagatcg gag		
<210> 779		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 779		
cgtcagctgcg gtcgctgcg t		
<210> 780		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 780		
ctgcctgcctga tccctgcctg g		
<210> 781		
<211> 21		
<212> DNA		
<213> Artificial Sequence		
<400> 781		
gcagagcctc cgaacctcgaa t		
<210> 782		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 782		
gcctaaagctggt agcgt		
<210> 783		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 783		
gcctagagctggt gagct		
<210> 784		
<211> 19		
<212> DNA		
<213> Artificial Sequence		
<400> 784		
gcatgagctggt gagct		
<210> 785		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 785		
gcatgagctggt gagct		
<210> 786		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 786		
gcatgagctggt gagct		
<210> 787		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 787		
gcatgagctggt gagct		
<210> 788		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 788		
gcatgagctggt gagct		
<210> 789		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 789		
gcatgagctggt gagct		
<210> 790		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 790		
gcatgagctggt gagct		
<210> 791		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 791		
gcatgagctggt gagct		
<210> 792		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 792		
gcatgagctggt gagct		
<210> 793		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 793		
gcatgagctggt gagct		
<210> 794		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 794		
gcatgagctggt gagct		
<210> 795		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 795		
gcatgagctggt gagct		
<210> 796		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 796		
gcatgagctggt gagct		
<210> 797		
<211> 15		
<212> DNA		
<213> Artificial Sequence		
<400> 797		

WO 01/2372	PCT/US90/26383	WO 01/2372	PCT/US90/26383
111	112		
<222> (8)...(10)	<213> Artificial Sequence		
<223> FTTG moiety attached at 3' end of sequence.	<400> 789		
<400> 784	agttgcact		10
tcaatgtga			
<210> 785	<210> 790		
<211> 10	<211> 8		
<212> DNA	<212> DNA		
<213> Artificial Sequence	<213> Artificial Sequence		
<220>	<400> 790		8
<221> misc_feature	tcttcgaa		
<222> (8)...(10)	<210> 791		
<223> FTTG moiety attached at 3' end of sequence.	<211> 8		
<400> 785	<212> DNA		
tcaatgtga	<213> Artificial Sequence		
<210> 786	<400> 791		8
<211> 10	tcaacgpc		
<212> DNA			
<213> Artificial Sequence	<210> 792		
<220>	<211> 19		
<221> misc_feature	<212> DNA		
<222> (8)...(10)	<213> Artificial Sequence		
<223> Blotin moiety attached at 3' end of sequence.	<400> 792		19
<400> 786	coattgtcgt cgtgtgct		
tcaacgtga			
<210> 787	<210> 793		
<211> 10	<211> 18		
<212> DNA	<212> DNA		
<213> Artificial Sequence	<213> Artificial Sequence		
<220>	<400> 793		18
<221> misc_feature	gttttttat atttggg		
<222> (8)...(10)	<210> 794		
<223> Blotin moiety attached at 3' end of sequence.	<211> 23		
<400> 787	<212> DNA		
gcattattgc	<213> Artificial Sequence		
<210> 788	<400> 794		23
<211> 10	tttttgttg tggtttgtc gtt		
<212> DNA	<210> 795		
<213> Artificial Sequence	<211> 12		
<220>	<212> DNA		
<221> misc_feature	<213> Artificial Sequence		
<222> (8)...(10)	<400> 795		12
<223> FTTG moiety attached at 3' end of sequence.	ttggggggggg tt		
<400> 788	<210> 796		
gcattattgc	<211> 13		
<210> 789	<212> DNA		
<211> 10	<213> Artificial Sequence		
<212> DNA	<400> 796		13
	gggggttgggg gtt		

W/O 01/29/97 113 PCT/US90/26383

<210> 797
<211> 17
<212> DNA
<213> Artificial Sequence

<400> 797
ggtgggtgag gttctgg 17

<210> 798
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (1)...(3)
<223> Blottn moiety attached at 5' end of sequence.

<221> modified_base
<222> (6)...(6)
<223> n5c

<400> 798
ggagangctc gactctgcat 20

<210> 799
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 799
tcacagctta cgttaacgct 20

<210> 800
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (1)...(3)
<223> Blottn moiety attached at 5' end of sequence.

<221> modified_base
<222> (8)...(8)
<223> n5c

<400> 800
ggagcaangtc gactctcat 20

<210> 801
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (1)...(3)
<223> Blottn moiety attached at 5' end of sequence.

<221> modified_base

W/O 01/29/97 114 PCT/US90/26383

<222> (6)...(6)
<223> n5c

<400> 801
ggagangctc cagctctgat 20

<210> 802
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (3)...(3)
<223> n5c

<221> misc_feature
<222> (8)...(10)
<223> Blottn moiety attached at 3' end of sequence.

<400> 802
tcangctga 10

<210> 803
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (2)...(2)
<223> n5c

<221> misc_feature
<222> (8)...(10)
<223> Blottn moiety attached at 3' end of sequence.

<400> 803
gnaactctgc 10

<210> 804
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 804
tgcgtctttt gtcgtttcgc gctt 24

<210> 805
<211> 22
<212> DNA
<213> Artificial Sequence

<400> 805
ctgcgtctgc aatttaactgc tg 22

<210> 806
<211> 20
<212> DNA
<213> Artificial Sequence

WO 01/21971	PCT/US00/25393	115	WO 01/21971	PCT/US00/25393	116
<400> 806 tcatgagct tctgtgctt	20		<212> DNA <213> Artificial Sequence		
<210> 807 <211> 28 <212> DNA <213> Artificial Sequence			<400> 814 tgatcagct ct		12
<400> 807 tgatgagct gatactgtao acagctct	28		<210> 815 <211> 20 <212> DNA <213> Artificial Sequence		
<210> 808 <211> 20 <212> DNA <213> Artificial Sequence			<400> 815 tgatgagct acacagctct		20
<400> 808 tgatgagct acacagctct			<210> 816 <211> 20 <212> DNA <213> Artificial Sequence		
<210> 809 <211> 12 <212> DNA <213> Artificial Sequence	20		<400> 816 ggcgaagctg gaccttcacat		20
<400> 809 tgatcagct ct			<210> 817 <211> 32 <212> DNA <213> Artificial Sequence		
<210> 810 <211> DNA <213> Artificial Sequence	12		<400> 817 ttaaagctaa agttaagctt aagcttaagct tt		32
<400> 810 tgatcagct			<210> 818 <211> 20 <212> DNA <213> Artificial Sequence		
<210> 811 <211> 20 <212> DNA <213> Artificial Sequence	8		<400> 818 ggcgaagctg gaccttcagat		20
<400> 811 aacacacacac cccccccccc			<210> 819 <211> 25 <212> DNA <213> Artificial Sequence		
<210> 812 <211> 12 <212> DNA <213> Artificial Sequence	20		<400> 819 gtccacattt cccagcggag gaact		25
<400> 812 cccccccccc cc			<210> 820 <211> 25 <212> DNA <213> Artificial Sequence		
<210> 813 <211> 8 <212> DNA <213> Artificial Sequence	12		<400> 820 ctagcagctg agctaatcaa gctag		25
<400> 813 cccccccc			<210> 821 <211> 25 <212> DNA <213> Artificial Sequence		
<210> 814 <211> 12	8		<400> 821 ctagcttgat gacgtacgcc gctag		25

W/O 01/29/97

117

PCYU1500/2533

<210> 822
<211> 16
<212> DNA
<213> Artificial Sequence
<400> 822
cggtgcagct cctcaaa
<210> 823
<211> 8
<212> DNA
<213> Artificial Sequence
<400> 823
ctgcagctg
<210> 824
<211> 10
<212> DNA
<213> Artificial Sequence
ctgcagctcaat
<400> 824
<210> 825
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 825
attcgatcag ggcggggcga g
<210> 826
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 826
ctgcgcgcgc cccgacgcga t
<210> 827
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 827
gactgcagct agcct
<210> 828
<211> 26
<212> DNA
<213> Artificial Sequence
<400> 828
ctagcgctcg acgtcacaaa gctcagc
<210> 829
<211> 26
<212> DNA
<213> Artificial Sequence

16

8

10

21

21

15

26

W/O 01/29/97

118

PCYU1500/2533

<400> 829
ctagcttat gcgcgcagcc gctcagc
<210> 830
<211> 26
<212> DNA
<213> Artificial Sequence
<400> 830
ctagcgctcg agctcacaaa gctcagc
<210> 831
<211> 25
<212> DNA
<213> Artificial Sequence
ctagcgctcg agctcacaaa gctcagc
<400> 831
<210> 832
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 832
tccacacagct ggtctatgct
<210> 833
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 833
ggagacgaaa gactctcatcaa taag
<210> 834
<211> 26
<212> DNA
<213> Artificial Sequence
<400> 834
tctcaaaaac atcctatctct aacacct
<210> 835
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 835
agctcaacgct cagcgc
<210> 836
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 836
ctaaagctcg taagcgtaact ggttc
<210> 837

26

26

25

20

24

26

15

24

120 27

tcagacacag tgcgcggtg ttcctga
 <210> 845
 <211> 27
 <212> DNA
 <213> Artificial Sequence

27

tcaggacac ccgacacgt ggtctga
 <400> 845
 <210> 846
 <211> 19
 <212> DNA
 <213> Artificial Sequence

19

catttcacg atttoca
 <400> 846
 <210> 847
 <211> 19
 <212> DNA
 <213> Artificial Sequence

19

ttctctctg ccagcgt
 <400> 847
 <210> 848
 <211> 19
 <212> DNA
 <213> Artificial Sequence

19

tgtctctctg tgcagcgt
 <400> 848
 <210> 849
 <211> 25
 <212> DNA
 <213> Artificial Sequence

25

ataagcgac actgacgca gtttc
 <400> 849
 <210> 850
 <211> 25
 <212> DNA
 <213> Artificial Sequence

25

gaaatgctg ctagttctg ttat
 <400> 850
 <210> 851
 <211> 30
 <212> DNA
 <213> Artificial Sequence

30

tgccacaga gaaatttg ttctacag
 <400> 851
 <210> 852
 <211> 30
 <212> DNA

119

<211> 24
 <212> DNA
 <213> Artificial Sequence
 <400> 837
 ttaagccac taagctaac aacg

<210> 838
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <400> 838
 gatctagtga tgcacagcc ggttc

<210> 839
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <400> 839
 gatcgcgtg aotcatcact agtc

<210> 840
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 840
 tcacagcgt tctgtgct

<210> 841
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 841
 tcatgacgt cctgtgct

<210> 842
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 842
 tcacacagt ggtgtgct

<210> 843
 <211> 17
 <212> DNA
 <213> Artificial Sequence
 <400> 843
 ccacgtgac ctctagc

<210> 844
 <211> 27
 <212> DNA
 <213> Artificial Sequence
 <400> 844

WO 01/2971

121

PCYUS00/26383

<213> Artificial Sequence
 <400> 852
 ctgatacgaac caaatttcc tctttgcca
 <210> 853
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 853
 tttaggttag gttcaggttt
 <210> 854
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 854
 tccatgagct tctttagtct
 <210> 855
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 855
 aaaaactgaac gtccaaaataa
 <210> 856
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 856
 aaaaactgaac gtccggggggg
 <210> 857
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 857
 ggggactgaac ctccggggggg
 <210> 858
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <400> 858
 ctgagctgaac gtccaaacagc tagt
 <210> 859
 <211> 30
 <212> DNA
 <213> Artificial Sequence
 <400> 859
 tctgaagcca tctgaacttg gttcgaagttat

30

20

20

20

20

20

24

30

WO 01/2971

122

PCYUS00/26383

<210> 860
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <400> 860
 ggaattagca ategattctag aagttc
 <210> 861
 <211> 30
 <212> DNA
 <213> Artificial Sequence
 <400> 861
 tttaactctt ataaaacataa ctaaaaataa
 <210> 862
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 862
 ggcgtttttt ttgcg
 <210> 863
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <400> 863
 atactcaatc aaaaactcaa caaa
 <210> 864
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <400> 864
 tctatcgaag gttggtctctg tttag
 <210> 865
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> also feature
 <222> (1)...(3)
 <223> Biotin moiety attached at 5' end of sequence.
 <400> 865
 tccatgagct tctttagtct
 <210> 866
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> also feature
 <222> (1)...(3)

25

30

15

24

24

20

123
 <22> Biotin moiety attached at 5' end of sequence.
 <400> 866
 tocatgagct tocatgagct 20
 <210> 867
 <211> 13
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc_feature
 <222> (11)...(13)
 <223> PTC moiety attached at 3' end of sequence.
 <221> misc_feature
 <222> (0)...(0)
 <223> Bsp phosphodiester backbone.
 <400> 867
 ttttttttt ttt 13
 <210> 868
 <211> 13
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> misc_feature
 <222> (11)...(13)
 <223> Biotin moiety attached at 3' end of sequence.
 <221> misc_feature
 <222> (0)...(0)
 <223> Bsp phosphodiester backbone with phosphodiester on 3' end.
 <400> 868
 ttttttttt ttt 13
 <210> 869
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <400> 869
 ctagcttgat gagctagac gctag
 <210> 870
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <400> 870
 ttcaattgc ttgcgtgta gctaa
 <210> 871
 <211> 20
 <212> DNA
 <213> Artificial Sequence

124
 <400> 871
 tocatgagct tocatgagct 20
 <210> 872
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <400> 872
 ctagcggcty agtcatcag tctag 25
 <210> 873
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 873
 tgcctagctgt gctgtacct 20
 <210> 874
 <211> 23
 <212> DNA
 <213> Artificial Sequence
 <400> 874
 atgctaaag agctacatt gca 23
 <210> 875
 <211> 23
 <212> DNA
 <213> Artificial Sequence
 <400> 875
 tgcattgta cgtccttag cat 23
 <210> 876
 <211> 31
 <212> DNA
 <213> Artificial Sequence
 <400> 876
 gtaggggact ttcgagctc gagatcctat g 31
 <210> 877
 <211> 31
 <212> DNA
 <213> Artificial Sequence
 <400> 877
 outagatct cagctcggg agtccctta c 31
 <210> 878
 <211> 22
 <212> DNA
 <213> Artificial Sequence
 <400> 878
 ctgtcagga ctgcggtaa gg 22
 <210> 879
 <211> 27

W0 01/29/72
125
PCT/US90/26383

<210> DNA
<213> Artificial Sequence

<400> 879
catatcactg gaattctac tccctcgc
<210> 880
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 880
ctccagctcc aagaaagaa g
<210> 881
<211> 21
<212> DNA
<213> Artificial Sequence

<400> 881
gaagttctcg gtaagctctc g
<210> 882
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 882
tcctcgccttc gtcgtcttcg gctt
<210> 883
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 883
tcgtcgtcttc gtcgtcttcg gctt
<210> 884
<211> 23
<212> DNA
<213> Artificial Sequence

<400> 884
tcgtcgtcttc tcgtcgtctc gctt
<210> 885
<211> 22
<212> DNA
<213> Artificial Sequence

<400> 885
tcctgacgctt cgcgcgcgcgc cc
<210> 886
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 886
tcgtcgtcttc gtcgtcttcg gctt

W0 01/29/72
126
PCT/US90/26383

<210> 887
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 887
tcctcgcctt tcctcgcctt
<210> 888
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 888
tcgtcgtcttc gtcgtcttcg cgtt
<210> 889
<211> 26
<212> DNA
<213> Artificial Sequence

<400> 889
tcgtcgtcttc cgtcgtcttc gtcgtt
<210> 890
<211> 27
<212> DNA
<213> Artificial Sequence

<400> 890
tcgtcgtcgt ctcgtcgttc tcgcctt
<210> 891
<211> 25
<212> DNA
<213> Artificial Sequence

<400> 891
ttcgcgtctt tcgtcgtcttc tcgtt
<210> 892
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 892
tcctgacgcgg gaagc
<210> 893
<211> 15
<212> DNA
<213> Artificial Sequence

<400> 893
tcctgacgcgc gaagc
<210> 894
<211> 15
<212> DNA
<213> Artificial Sequence

<211> 20
<212> DNA
<213> Artificial Sequence

20

902 <400>

<210> 903
<211> 20
<212> DNA
<213> Artificial Sequence

20

<400> 903
гсгвсгтсгс гсгсгсгсгс

<210> 904
<211> 15
<212> DNA
<213> Artificial Sequence

15

tcgtcgtctgt ctccg
 <400> 904

<210> 905
<211> 20
<212> DNA
<213> Artificial Sequence

20

<400> 905
ctgtgggggtt ctggttcttg

<210> 906
<211> 20
<212> DNA
<213> Artificial Sequence

20

906 > 400

<210> 907
<211> 21
<212> DNA
<213> Artificial Sequence

21

<400> 907

<210> 908
<211> 22
<212> DNA
<213> Artificial Sequence

22

<400> 908
atctctctctct atctctctct ct

<210> 909
<211> 20
<212> DNA
<213> Artificial Sequence

606-5007

WFO 01/23/71
129
20
gvggtcgagc tccagggggg
<210> 910
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 910
atctatctat atctatctat at
<210> 911
<211> 27
<212> DNA
<213> Artificial Sequence
<400> 911
tttttttt tttttttt tttttt
<210> 912
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 912
tttttttt tttttttt c
<210> 913
<211> 18
<212> DNA
<213> Artificial Sequence
<400> 913
tttttttt tttttttt
<210> 914
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 914
gctcaggggg agggc
<210> 915
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 915
gctcaggggc agggg
<210> 916
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 916
gctcaggggg gagct
<210> 917
<211> 20
<212> DNA

PCT/US80/26383

WFO 01/23/71
130
20
<213> Artificial Sequence
<400> 917
atggaagtc cagggggc
<210> 918
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 918
atggaactcg gggggggc
<210> 919
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 919
atggaagtc cagggggc
<210> 920
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 920
gagaaagggg gacctggat
<210> 921
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 921
gagaaagggg gacctcgaat
<210> 922
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 922
gagaaagggg cagactgat
<210> 923
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 923
tccatggggg gacctgatgc
<210> 924
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 924
tccatggggg gacctgatgc
<210> 925
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 925
tccatggggg gacctgatgc

PCT/US80/26383

<400> 932
tccatgagg tgggatgct
<210> 933
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 933
tccatggggg tccatgct
<210> 934
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 934
tccatggggg cccatgct
<210> 935
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 935
tccatggggg cccatgct
<210> 936
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 936
tccatggggg tccatgct
<210> 937
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 937
tccatggggg cccatgct
<210> 938
<211> 14
<212> DNA
<213> Artificial Sequence

<400> 938
gctagaggga gctg
<210> 939
<211> 18
<212> DNA
<213> Artificial Sequence

<400> 939
tttttttttt tttttt
<210> 940
<211> 21

<210> 925
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 925
tccatgagg gctgctgct
<210> 926
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 926
atggactctc cggggctctc
<210> 927
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 927
atggaggctc cggggctctc
<210> 928
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 928
atggactctc cggggctctc
<210> 929
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 929
atggaggctc cggggctctc
<210> 930
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 930
atggactctg cggggctctc
<210> 931
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 931
tccatgagg tgggatgct
<210> 932
<211> 20
<212> DNA
<213> Artificial Sequence

WFO 01/29/72

PC70500/26383

133

<212> DNA
<213> Artificial Sequence
<220>
<221> misc difference
<222> (2) .. (2)
<223> m 1a a or c
<231> misc difference
<232> (18) .. (18)
<233> m 1a a or c
<400> 940
gggttcaagc ttgaggggg g
<210> 941
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 941
gggggggtcg ttgagggggg g
<210> 942
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 942
tcgtggttc ccccccgc
<210> 943
<211> 25
<212> DNA
<213> Artificial Sequence
<400> 943
ttgggggggtc tttttttt tttt
<210> 944
<211> 23
<212> DNA
<213> Artificial Sequence
<400> 944
tttaatttc aaatttaa ata
<210> 945
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 945
ttgggtttt tgggtttt ttgg
<210> 946
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 946

24

23

25

20

21

21

WFO 01/29/72

PC70500/26383

134

ttcccttcc cccctttcc atcc
<210> 947
<211> 21
<212> DNA
<213> Artificial Sequence
<220>
<221> misc difference
<222> (21) .. (21)
<223> a 1a g or c
<400> 947
gggttcacgc atgaggggg g
<210> 948
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 948
tccatgacgc tccatgacgt
<210> 949
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 949
tccatgacgc tccatgacgt
<210> 950
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 950
tccatgacgc tccatgacgt
<210> 951
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 951
tccatgacgc tccatgacgt
<210> 952
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 952
tccatgacgc tccatgacgt
<210> 953
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 953

24

20

20

20

20

21

WO 01/22972

PCT/US02/3393

135 20

tccatgaagt tccatgaagtt
<210> 954
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 934
tccatgaagt tccatgaagtt
<210> 935
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 955
tccatgaagt tccatgaagtt
<210> 936
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 956
tccatgaagt tccatgaagtt
<210> 937
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 957
tccatgaagt tccatgaagtt
<210> 938
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 958
tccatgaagt tccatgaagtt
<210> 959
<211> 19
<212> DNA
<213> Artificial Sequence

<400> 959
gccccgcagct cgtccggggg
<210> 960
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 960
gggggttcgta agacggggggg
<210> 961
<211> 24
<212> DNA

20

<210> 962
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 963
cccccccccc cccccccccccc
<210> 964
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 965
tccatgaagt tccatgaagtt
<210> 966
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 967
tccatgaagt tccatgaagtt
<210> 968
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 968
tccatgaagt tccatgaagtt
<210> 969
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 969
tccatgaagt tccatgaagtt
<210> 970
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 971
tccatgaagt tccatgaagtt
<210> 972
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 973
tccatgaagt tccatgaagtt
<210> 974
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 975
tccatgaagt tccatgaagtt
<210> 976
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 977
tccatgaagt tccatgaagtt
<210> 978
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 979
tccatgaagt tccatgaagtt
<210> 980
<211> 24
<212> DNA
<213> Artificial Sequence

20

<210> 981
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 982
tccatgaagt tccatgaagtt
<210> 983
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 984
tccatgaagt tccatgaagtt
<210> 985
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 986
tccatgaagt tccatgaagtt
<210> 987
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 988
tccatgaagt tccatgaagtt
<210> 989
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 989
tccatgaagt tccatgaagtt
<210> 990
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 991
tccatgaagt tccatgaagtt
<210> 992
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 993
tccatgaagt tccatgaagtt
<210> 994
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 995
tccatgaagt tccatgaagtt
<210> 996
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 997
tccatgaagt tccatgaagtt
<210> 998
<211> 24
<212> DNA
<213> Artificial Sequence

<400> 999
tccatgaagt tccatgaagtt
<210> 1000
<211> 24
<212> DNA
<213> Artificial Sequence

W0 01/23/72
137
PCT/US80/24383
<210> 969
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 969
gggtcacagc tctagggggg
<210> 970
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 970
gggtcacagc tctagggggg
<210> 971
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 971
tgctgcttc ccccccac
<210> 972
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 972
gggtcacagc acgtcggggg
<210> 973
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 973
gggtcacagc acgtcggggg
<210> 974
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 974
gggtcacagc tactcgagg gggg
<210> 975
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 975
gggtcacagc acgtcgagg ggg
<210> 976
<211> 19
<212> DNA
<213> Artificial Sequence

W0 01/23/72
138
PCT/US80/24383
<400> 976
gggtcacagc cgaagggggg
<210> 977
<211> 19
<212> DNA
<213> Artificial Sequence
<400> 977
gggtcacagc tcaagggggg
<210> 978
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 978
gggtcacagc aacgtcgagg ggg
<210> 979
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 979
gggtcacagc gtcagggggg
<210> 980
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 980
gggtcacagc gaacgggggg ggg
<210> 981
<211> 22
<212> DNA
<213> Artificial Sequence
<400> 981
gggtcacagc gaacgtcgagg ggg
<210> 982
<211> 10
<212> DNA
<213> Artificial Sequence
<400> 982
tcaactctga
<210> 983
<211> 10
<212> DNA
<213> Artificial Sequence
<400> 983
tcaagctga
<210> 984
<211> 12

WO 01/2972	PCT/US00/2383	139	WO 01/2972	PCT/US00/2383	140
<212> DNA			<210> 992		
<213> Artificial Sequence			<211> 8		
			<212> DNA		
<400> 984	12		<213> Artificial Sequence		
tcacgatcgt ga					
			<400> 992		
<210> 995			tcaacggt		
<211> 12					
<212> DNA			<210> 993		
<213> Artificial Sequence			<211> 20		
			<212> DNA		
<400> 985	12		<213> Artificial Sequence		
tcacgatcgt ga					
			<400> 993		
<210> 996			tcataacgg tccgatgct		
<211> 20					
<212> DNA			<210> 994		
<213> Artificial Sequence			<211> 20		
			<212> DNA		
<400> 986	20		<213> Artificial Sequence		
ggggagacat gctcgggggg					
			<400> 994		
<210> 987			tcataacgg tccacgggt		
<211> 20					
<212> DNA			<210> 995		
<213> Artificial Sequence			<211> 20		
			<212> DNA		
<400> 987	20		<213> Artificial Sequence		
gggggggggg gggggggggg					
			<400> 995		
<210> 988			gggggacgat cgtcgggggg		
<211> 22					
<212> DNA			<210> 986		
<213> Artificial Sequence			<211> 20		
			<212> DNA		
<400> 988	22		<213> Artificial Sequence		
gggggacgat atcgtcgggg gg					
			<400> 986		
<210> 989			gggggacgat cgtcgggggg		
<211> 22					
<212> DNA			<210> 987		
<213> Artificial Sequence			<211> 21		
			<212> DNA		
<400> 989	22		<213> Artificial Sequence		
gggggacgac ctcgtcgggg gg					
			<400> 987		
<210> 990			gggggggacga tgcgtcgggg g		
<211> 22					
<212> DNA			<210> 988		
<213> Artificial Sequence			<211> 21		
			<212> DNA		
<400> 990	22		<213> Artificial Sequence		
gggggacgag ctcgtcgggg gg					
			<400> 988		
<210> 991			gggggacgat cgtcgggggg g		
<211> 20					
<212> DNA			<210> 989		
<213> Artificial Sequence			<211> 12		
			<212> DNA		
<400> 991	20		<213> Artificial Sequence		
gggggacgta cgtcgggggg					

WO 01/2972
143
PCT/US90/26383
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1014
ccgactgactg atctggttgg
<210> 1015
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1015
ctatagagctc gctccagctg
<210> 1016
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1016
catgtctggagc gaggctctata
<210> 1017
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1017
tcgtcgttggg gattcaagctc
<210> 1018
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1018
gagcttactcc cctcacacacca
<210> 1019
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1019
gaccttggatc ttccgttggga
<210> 1020
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1020
ttgactcttcc ttctgcctct
<210> 1021
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1021

WO 01/2972
144
PCT/US90/26383
atgctgtgac ccagcggctaa
<210> 1022
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1022
accgattcag cggaaagttca
<210> 1023
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1023
tccatgtgagc tccctggagct
<210> 1024
<211> 24
<212> DNA
<213> Artificial Sequence
<400> 1024
ggagagaccg atgagctcat ctgg
<210> 1025
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1025
accacagagac agcagagcaga
<210> 1026
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1026
gagctctgagc ttccgctgaga
<210> 1027
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1027
tcgttaccct tgcagcggct
<210> 1028
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1028
ctggagacct agccaaagat
<210> 1029
<211> 20
<212> DNA

WO 01/23972
 147
 <210> 1044
 <211> 24
 <212> DNA
 <213> Artificial Sequence
 <400> 1044
 ggggcagcg taagtcaggg gggg
 <210> 1045
 <211> 22
 <212> DNA
 <213> Artificial Sequence
 <400> 1045
 ggggacgcgc accgcgcggg gg
 <210> 1046
 <211> 18
 <212> DNA
 <213> Artificial Sequence
 <400> 1046
 gggtcgaagt cgggggggg
 <210> 1047
 <211> 18
 <212> DNA
 <213> Artificial Sequence
 <400> 1047
 ggggcagcg tcaggggg
 <210> 1048
 <211> 22
 <212> DNA
 <213> Artificial Sequence
 <400> 1048
 ggggacgcgc taagtcaggg gg
 <210> 1049
 <211> 19
 <212> DNA
 <213> Artificial Sequence
 <400> 1049
 ggggacgcgc aagtcggggg
 <210> 1050
 <211> 34
 <212> DNA
 <213> Artificial Sequence
 <400> 1050
 ggaactctcg aagtcagcag cggagagctc tgaat
 <210> 1051
 <211> 32
 <212> DNA
 <213> Artificial Sequence

PCT/US00/2583
 148
 <400> 1051
 cggactctcc atgagagctc tgaactctt gg
 <210> 1052
 <211> 35
 <212> DNA
 <213> Artificial Sequence
 <400> 1052
 cggactctcc atgagagctc ttggagcagatg tgaac
 <210> 1053
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 1053
 tccgcagagt tgaagt
 <210> 1054
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1054
 ggggggcgct cgttcggggg
 <210> 1055
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1055
 gggggagatgat tgcgcggggg
 <210> 1056
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (7)...(7)
 <223> m5c
 <221> modified base
 <222> (11)...(11)
 <223> m5c
 <400> 1056
 gggggagatgat ngttcggggg
 <210> 1057
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1057
 gggggagccta gcttcggggg
 <210> 1058

WO 01/23972
 148
 <400> 1051
 cggactctcc atgagagctc tgaactctt gg
 <210> 1052
 <211> 35
 <212> DNA
 <213> Artificial Sequence
 <400> 1052
 cggactctcc atgagagctc ttggagcagatg tgaac
 <210> 1053
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 1053
 tccgcagagt tgaagt
 <210> 1054
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1054
 ggggggcgct cgttcggggg
 <210> 1055
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1055
 gggggagatgat tgcgcggggg
 <210> 1056
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (7)...(7)
 <223> m5c
 <221> modified base
 <222> (11)...(11)
 <223> m5c
 <400> 1056
 gggggagatgat ngttcggggg
 <210> 1057
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1057
 gggggagccta gcttcggggg
 <210> 1058

PCT/US00/2583
 148
 <400> 1051
 cggactctcc atgagagctc tgaactctt gg
 <210> 1052
 <211> 35
 <212> DNA
 <213> Artificial Sequence
 <400> 1052
 cggactctcc atgagagctc ttggagcagatg tgaac
 <210> 1053
 <211> 15
 <212> DNA
 <213> Artificial Sequence
 <400> 1053
 tccgcagagt tgaagt
 <210> 1054
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1054
 ggggggcgct cgttcggggg
 <210> 1055
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1055
 gggggagatgat tgcgcggggg
 <210> 1056
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> modified base
 <222> (7)...(7)
 <223> m5c
 <221> modified base
 <222> (11)...(11)
 <223> m5c
 <400> 1056
 gggggagatgat ngttcggggg
 <210> 1057
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <400> 1057
 gggggagccta gcttcggggg
 <210> 1058

tcacgggaatt ctctcaggtt tttt

<210> 1066
<211> 20
<212> DNA
<213> Artificial Sequence

tcacaaactt ctctcaatt

<400> 1066
<210> 1067
<211> 24
<212> DNA
<213> Artificial Sequence

tactactttt atacttttat acct

<400> 1067
<210> 1068
<211> 24
<212> DNA
<213> Artificial Sequence

tgtgtgtgtg tgtgtgtgtg tgtg

<400> 1068
<210> 1069
<211> 25
<212> DNA
<213> Artificial Sequence

tgtgtgtgtg tgtgtgtgtg tgtg

<400> 1069
<210> 1070
<211> 27
<212> DNA
<213> Artificial Sequence

ggctccgggg agggaaattt tgtctat

<400> 1070
<210> 1071
<211> 19
<212> DNA
<213> Artificial Sequence

gggcgcgatcg tcgggggggg

<400> 1071
<210> 1072
<211> 20
<212> DNA
<213> Artificial Sequence

gggtcgtcga cpa-gggggggg

<400> 1072
<210> 1073
<211> 19
<212> DNA

ggttcctttg gtcattgtct

<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1058
<210> 1059
<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1059
<210> 1060
<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1060
<210> 1061
<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1061
<210> 1062
<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1062
<210> 1063
<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1063
<210> 1064
<211> 20
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1064
<210> 1065
<211> 24
<212> DNA
<213> Artificial Sequence

ggttcctttg gtcattgtct

<400> 1065
<210> 1066
<211> 20
<212> DNA
<213> Artificial Sequence

W01/25972	PCT/US00/2583	W01/25972	PCT/US00/2583
151	152		
<213> Artificial Sequence	<210> 1081		
<400> 1073	<211> 15		
ggtcgtcgcgc ggcgcgcgcgc	<212> DNA		
<210> 1074	<213> Artificial Sequence		
<211> 20	<400> 1081		
<212> DNA	tcctgcgcgc ggaagc		15
<213> Artificial Sequence	<210> 1082		
<400> 1074	<211> 15		
gggtcgtcgt cgtcgcgcgcgc	<212> DNA		
<210> 1075	<213> Artificial Sequence		
<211> 20	<400> 1082		
<212> DNA	tcctgcgcgc ggaagc		15
<213> Artificial Sequence	<210> 1083		
<400> 1075	<211> 15		
gggcgcgcgc gtcgcgcgcgc	<212> DNA		
<210> 1076	<213> Artificial Sequence		
<211> 20	<400> 1083		
<212> DNA	tcctgcgcgc ggaagc		15
<213> Artificial Sequence	<210> 1084		
<400> 1076	<211> 15		
gggcgcgcgc tcgtgcgcgcgc	<212> DNA		
<210> 1077	<213> Artificial Sequence		
<211> 27	<400> 1084		
<212> DNA	tcctgcgcgc caagc		15
<213> Artificial Sequence	<210> 1085		
<400> 1077	<211> 15		
gggcgcgcgc tcgcgcgcgcgc	<212> DNA		
<210> 1078	<213> Artificial Sequence		
<211> 21	<400> 1085		
<212> DNA	tcctgcgcgc tcagc		15
<213> Artificial Sequence	<210> 1086		
<400> 1078	<211> 15		
gggcgcgcgc ggtcgcgcgcgc	<212> DNA		
<210> 1079	<213> Artificial Sequence		
<211> 21	<400> 1086		
<212> DNA	tcctgcgcgc aaagc		15
<213> Artificial Sequence	<210> 1087		
<400> 1079	<211> 15		
gggcgcgcgc tcgtgcgcgcgc	<212> DNA		
<210> 1080	<213> Artificial Sequence		
<211> 23	<400> 1087		
<212> DNA	tcgcgcgcgc ggaagc		15
<213> Artificial Sequence	<210> 1088		
<400> 1080	<211> 15		
tcgtcgtcgt cgtcgcgcgcgc	<212> DNA		
	<213> Artificial Sequence		

<400> 1095
aaaaaaaaa aaaaaaaaaa aaaaaaaaaa
<210> 1096
<211> 30
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence
<400> 1096
ccccccccc ccccccccc ccccccccc
<210> 1097
<211> 30
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence
<400> 1097
cgcgcgcgc cgcgcgcgc cgcgcgcgc
<210> 1098
<211> 12
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence
<400> 1098
gattttatcg tc
<210> 1099
<211> 12
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence
<400> 1099
tcgatttttc ga
<210> 1100
<211> 12
<212> DNA
<213> Artificial Sequence
<400> 1100
tcatttttat ga
<210> 1101
<211> 12
<212> DNA
<213> Artificial Sequence
<400> 1101
gttttttacy ac

30

30

30

12

12

12

12

<400> 1088
tcgggggggg gaagt
<210> 1089
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 1089
tcgggggggg gaagt
<210> 1090
<211> 15
<212> DNA
<213> Artificial Sequence
<400> 1090
gggggggggt ggggg
<210> 1091
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1091
gggggttttt ttgtggggg
<210> 1092
<211> 20
<212> DNA
<213> Artificial Sequence
<400> 1092
ggggggcccc cccggggggg
<210> 1093
<211> 21
<212> DNA
<213> Artificial Sequence
<400> 1093
gggggttgtg ttgtggggg g
<210> 1094
<211> 30
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence
<400> 1094
tttttttttt tttttttttt tttttttt
<210> 1095
<211> 30
<212> DNA
<213> Artificial Sequence
<220>
<223> Synthetic Sequence

15

15

15

20

20

21

30

<210> 1102	
<211> 12	
<212> DNA	
<213> Artificial Sequence	
<400> 1102	
tcatttttc ga	12
<210> 1103	
<211> 12	
<212> DNA	
<213> Artificial Sequence	
<400> 1103	
acgtttttac gc	12
<210> 1104	
<211> 12	
<212> DNA	
<213> Artificial Sequence	
<400> 1104	
tcgttttttc ga	12
<210> 1105	
<211> 16	
<212> DNA	
<213> Artificial Sequence	
<400> 1105	
tcgttttttc cgtcga	16
<210> 1106	
<211> 14	
<212> DNA	
<213> Artificial Sequence	
<400> 1106	
aatttttttac cgtt	14
<210> 1107	
<211> 14	
<212> DNA	
<213> Artificial Sequence	
<400> 1107	
tcgttttttc aaga	14
<210> 1108	
<211> 14	
<212> DNA	
<213> Artificial Sequence	
<400> 1108	
acgttttttc acgt	14
<210> 1109	
<211> 13	
<212> DNA	
<213> Artificial Sequence	

<210> 1110	
<211> 12	
<212> DNA	
<213> Artificial Sequence	
<400> 1110	
tcatttttc ga	12
<210> 1111	
<211> 12	
<212> DNA	
<213> Artificial Sequence	
<400> 1111	
acgttttttc gc	12
<210> 1112	
<211> 12	
<212> DNA	
<213> Artificial Sequence	
<400> 1112	
tcgttttttc ga	12
<210> 1113	
<211> 16	
<212> DNA	
<213> Artificial Sequence	
<400> 1113	
tcgttttttc cgtcga	16
<210> 1114	
<211> 14	
<212> DNA	
<213> Artificial Sequence	
<400> 1114	
aatttttttac cgtt	14
<210> 1115	
<211> 14	
<212> DNA	
<213> Artificial Sequence	
<400> 1115	
tcgttttttc aaga	14
<210> 1116	
<211> 14	
<212> DNA	
<213> Artificial Sequence	
<400> 1116	
acgttttttc acgt	14
<210> 1117	
<211> 13	
<212> DNA	
<213> Artificial Sequence	

V502/2E488

WO 01/2972	PCT/US0076383	158	159	PCT/US0076383
<210> 1125			<400> 1132	
<211> 10			gacgatgctc tc	12
<212> DNA			<210> 1133	
<213> Artificial Sequence			<211> 12	
			<212> DNA	
<400> 1125	10		<213> Artificial Sequence	
acgtgtacgt				
<210> 1126			<400> 1133	12
<211> 10			gatgagctgc tc	
<212> DNA			<210> 1134	
<213> Artificial Sequence			<211> 10	
			<212> DNA	
<400> 1126	10		<213> Artificial Sequence	
tcgtgtacga			<400> 1134	10
<210> 1127			gatgtacgtc	
<211> 14			<210> 1135	
<212> DNA			<211> 8	
<213> Artificial Sequence			<212> DNA	
			<213> Artificial Sequence	
<400> 1127	14			
tcgtgtacgc tcga			<400> 1135	8
<210> 1128			atgatcgt	
<211> 12			<210> 1136	
<212> DNA			<211> 12	
<213> Artificial Sequence			<212> DNA	
			<213> Artificial Sequence	
<400> 1128	12			
aatgttacgc tc			<400> 1136	12
<210> 1129			aacgatgtgc tt	
<211> 12			<210> 1137	
<212> DNA			<211> 10	
<213> Artificial Sequence			<212> DNA	
			<213> Artificial Sequence	
<400> 1129	12			
tcgtgttacgc ga			<400> 1137	10
<210> 1130			tcactgtgtgc	
<211> 12			<210> 1138	
<212> DNA			<211> 8	
<213> Artificial Sequence			<212> DNA	
			<213> Artificial Sequence	
<400> 1130	12			
acgtgttacgc gt			<400> 1138	8
<210> 1131			tcgtatgc	
<211> 11			<210> 1139	
<212> DNA			<211> 12	
<213> Artificial Sequence			<212> DNA	
			<213> Artificial Sequence	
<400> 1131	11			
gatgtatcgt c			<400> 1139	12
<210> 1132			actgtgtacgc gt	
<211> 12			<210> 1140	
<212> DNA				
<213> Artificial Sequence				

WO 01/25972

PCT/US90/24353

160

<211> 16
<212> DNA
<213> Artificial Sequence

16

topatgcga optcga
<400> 1140
<210> 1141
<211> 10
<212> DNA
<213> Artificial Sequence

<400> 1141
acgatgctcgt

10

<210> 1142
<211> 31
<212> DNA
<213> Artificial Sequence

<400> 1142
tcgaggaagat ccaggtcttcc cccaacccc c

31

<210> 1143
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<221> Synthetic Sequence

<400> 1143
gaagtc

6

<210> 1144
<211> 6
<212> DNA
<213> Artificial Sequence

<400> 1144
gtcgtc

6

<210> 1145
<211> 6
<212> DNA
<213> Artificial Sequence

<400> 1145
tcagtcgt

8

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)